PROCEDURES RECOMMENDED FOR THE STUDY OF BACTERIA,

WITH ESPECIAL REFERENCE TO GREATER UNIFORMITY IN THE DESCRIPTION AND DIFFERENTIATION OF SPECIES.

BEING THE REPORT OF A COMMITTEE OF AMERICAN BACTERIOLOGISTS TO THE COMMITTEE ON THE POLLUTION OF WATER SUPPLIES OF THE AMERICAN PUBLIC HEALTH ASSOCIATION.

Submitted at meeting held in Philadelphia, Pa., Sept. 1897

CONCORD, N. H.
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PREFACE.

At the meeting of the American Public Health Association in Montreal, Canada, in 1894, the Committee on the Pollution of Water Supplies closed its report with the suggestion of a co-operative investigation into the bacteriology of water supplies as a means of bringing order out of the chaotic state of the literature of water bacteria, and of throwing light from the bacteriological side on questions of practical sanitation. This suggestion[1] was approved by the Association and the Chairman of the Committee was authorized to build up a committee for collective bacteriological investigation. The bacteriologists promptly acceded to the proposition. They recognized that such an investigation would give an immense impetus to bacteriological work; that it would do much to clear away the confusion surrounding species, and to increase and systematize our knowledge; and that practical results might also be expected, particularly as regards the typhoid and colon bacilli, the unwholesomeness of water supplies and the means of lessening the prevalence of typhoid fever and diarrheal diseases. A sub-committee consisting of Professor J. George Adami, Dr. Wyatt Johnston, Mr. George W. Fuller, and myself, appointed to determine the methods of laboratory procedure to be adopted by the committee in the practical work of the investigation, found it impossible to formulate a satisfactory scheme of work until certain questions, mostly relating to technique, had been discussed fully and settled in accordance with the most advanced knowledge of the subjects concerned. An effort to effect this by correspondence developed so much variance in the practice of the different laboratories that it became needful to call a convention for a thorough discussion of the points at issue. The convention was held in the Academy of Medicine, New York city, June 21 and 22, 1895. Most of the prominent bacteriologists of the United States and Canada were present, but although the members were informed beforehand of the subjects that were to be brought up for settlement, and although full discussion was given to each under the chairmanship of Professor Welch of Johns Hopkins University, many of the points presented so much difficulty that the whole series was referred to a committee, with the understanding that the convention would accept its decision.

This committee consisted of

J. George Adami, McGill University, Chairman.
A. C. Abbott, University of Pennsylvania.
T. M. Cheesman, College Physicians and Surgeons, New York.
George W. Fuller, Louisville Water Company.

[1] For which credit is due to Dr. Wyatt Johnston.

W. T. Sedgwick, State Board of Health, Massachusetts.
Charles Smart, U. S. Army.
Theobald Smith, Harvard University.
W. H. Welch, Johns Hopkins University.

The committee met in New York city in February, 1896, to digest its materials and outline its report which was presented to the American Public Health Association at its meeting in Buffalo, New York, in September of that year. The report was subsequently withdrawn for further criticism and amendment, and was finally submitted for publication at the meeting of the Association in Philadelphia, Pennsylvania, September, 1897.

CHARLES SMART,
Deputy Surgeon General, U. S. Army.

INTRODUCTION.

As explained by Dr. Smart in the preface to this report, a convention of bacteriologists from the United States and Canada assembled in the city of New York, on June 21 and 22, 1895, in response to the invitation of a sub-committee of the Committee on the Pollution of Water Supplies of the American Public Health Association. The proceedings of this convention, including the papers read and their discussion, were published in the Journal of the American Public Health Association, October, 1895. These papers and discussions related mainly to technical procedures to be followed in the systematic study of bacteria with especial reference to their description and identification. There was general agreement of opinion as to the importance of securing greater precision and uniformity in the methods of studying and describing bacterial species. A committee of members of the convention was, therefore, appointed to prepare a report, to be presented to the Water Committee of the American Public Health Association, this report to contain recommendations concerning bacteriological methods based partly upon the deliberations of the convention and partly upon a wider study of the subject. The members selected for this committee were Drs. J. George Adami, William T. Sedgwick, George W. Fuller, Charles Smart, Alexander C. Abbott, T. M. Cheesman, Theobald Smith, and William H. Welch.

A first draft of a report was drawn up by Dr. Adami and submitted to the members of the committee, who made various suggestions. The final preparation of the report was undertaken by Dr. T. M. Cheesman, Instructor in Bacteriology in the College of Physicians and Surgeons, Columbia University, New York. The following statement by Dr. Adami well expresses the aims and manner of preparation of the report: "Naturally with a committee, the members of which are so widely scattered, it has been found impossible to hold frequent meetings, but at these meetings the members have found themselves singularly in accord upon everything relating to the main points at issue. Naturally, also, correspondence and the circulation of the report in its various stages have not been found entirely satisfactory in eliciting the opinions of every member upon matters of detail. But all these means accomplished much, and it was eventually found possible to place the final drafting of the recommendations in the hands of one member. We cannot sufficiently express our indebtedness to Dr. Cheesman for the amount of time, and indeed of independent work which he has devoted to this task.

"The recommendations thus do not indicate the previous procedure in all details of any single member of the committee, but are a concord of what has appeared to be the best in the methods and technique of all the members and of bacteriologists generally. To have indicated in the following pages wherein any single member found himself unable to accept in its entirety any one of the many recommendations would have

counteracted our main object, that, namely, of inducing uniformity and precision in procedure in the study and descriptions of species. Each member, therefore, to attain this object has voluntarily refrained from demanding that one or other method, to which from long employment he has become firmly attached, should be inserted in these pages. The committee freely admits that there may be other and better methods than those here detailed. It has, on the other hand, striven to recommend what in the present state of our knowledge would seem to be the best and most likely to gain acceptance. It does not demand of bacteriologists in general—it does not promise for its own members in particular—that these and only these methods shall be employed. It does but ask that *where new species are being studied for publication* the procedure here recommended be given a trial, and that, for the direction of other workers, where it has been employed a note be given to that effect, *e. g.*, 'cultures in broth (Method B. C.[1]) presented the following characters:'—or, 'save where otherwise indicated, the B. C. methods have been used.'

"In short, the committee recognizes fully that these recommendations must of necessity be provisional. It publishes them in the hope that by this act it will direct attention to the urgent need now existing for full and accurate descriptions of species of bacteria in which the items have been determined by methods common to the main body of workers, and as a consequence are capable of verification and control."

The report is not intended to be a complete treatise upon bacteriological technique. Its purpose is to make certain recommendations concerning methods to be pursued in the study of bacteria, with the view of securing greater uniformity and exactness in the determination and description of the characters of bacterial species. When one considers the difficulty, often the impossibility, of the identification of many bacterial species or varieties described in literature, in consequence of imperfections and carelessness in the determination and description of their characters, it is evident that the attainment of the purpose aimed at in this report is greatly to be desired.

The report deals especially with certain ordinary and fundamental procedures in bacteriological technique, and it does not attempt to cover fully the entire field. In a science so rapidly developing as bacteriology, it need scarcely be said that any attempt to present the best technical procedures can apply only to the existing state of the science, and that much will be added and much corrected in the near future. It is hoped that the recommendations in this report may prove useful to workers in bacteriology, and especially may lead to greater accuracy and fullness and uniformity in the determination and description of the characters of bacteria.

WILLIAM H. WELCH.

[1] Bacteriological Committee; the methods recommended by the Committee of American Bacteriologists.

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The various tests applicable for describing species of bacteria may be divided into two categories, as follows:

Necessary tests, and those which in the present condition of bacterial science may be regarded as optional.

The terms "necessary" and "optional" are here used with considerable hesitation, as many of the tests included among the optional are of importance and really necessary for the purposes of species differentiation in special cases. These tests, as grouped, however, are at the present time applicable to a great majority of the species known, those classed as necessary being of primary importance and of the greatest general utility.

NECESSARY INFORMATION AND TESTS.

Information with regard to the following features and properties of any species of bacteria that is being studied is held by the committee to be necessary and to form the indispensible basis for conclusions as to the characters and relationships of that species.

I. Source and habitat.

II. Morphological characters.
   1. Form.
   2. Dimensions.
   3. Manner of grouping and arrangement in the growths.
   4. Staining powers, (a) with watery dyes, (b) by Gram's method.
   5. Presence or absence of capsule.
   6. Presence or absence of flagella (motility).
   7. Spore formation and differentiation of spores from deposits and vacuoles within the cell.
   8. Tendency to pleomorphism.
   9. Involution and degeneration forms.

III. Biological characters.
  A. Cultural characteristics, mode of growth in and upon
    1. Nutrient broth.
    2. Gelatin plates (single colonies, surface and deep).
    3. Gelatin tubes.
    4. Agar plates (single colonies, surface and deep).
    5. Agar tubes.
    6. Potato.
    7. Milk.
    8. Blood serum.
  B. Biochemical features.
    1. Temperature relationship (activity of growth at 18°-22° C. and at 36°-38° C. and thermal death point).
    2. Relation to free oxygen (aërobic and anaërobic growth).
    3. Relation of growth to acidity and alkalinity of media.
    4. Action upon gelatin (presence or absence of liquefaction).
    5. Action upon proteids (milk and serum).
    6. Action upon carbohydrates (fermentation and gas formation).
    7. Action upon nitrates.
    8. Production of indol.
    9. Production of acid or alkali.
    10. Pigment formation.
    11. Development of odor.
  C. Pathogenesis.

OPTIONAL TESTS OF GENERAL USEFULNESS.

I. Morphological.
  1. Staining reactions with special stains.
  2. Study of flagella by special stains.
  3. Permanency of morphological characters after long-continued growth and successive transplantation upon artificial media.
  4. Photographic reproductions of isolated bacteria.
  5. Cover-glass impressions.

II. Physiological.
  A. Cultural characteristics, mode of growth in or upon
    1. Litmus gelatin.
    2. Loeffler's blood serum.
    3. Synthesized media.
    Photographic reproduction of characteristic cultures.
  B. Biochemical.
    1. Minimum, optimum, and maximum temperatures of growth.
    2. Growth in atmospheres of various inert gases (when anaerobic power of growth has been determined).

3. Optimum reaction of media and reaction limit, acid and alkaline (indicated by phenolphthalein).
4. Chemical properties and solubility of pigments produced and spectroscopic observations upon the pigments.

C. Pathogenesis.

1. Inoculation of various species of animals with minute study of the pathological changes produced.
2. Immunity-producing properties.
3. Agglutinating properties of specific sera.
4. Determination and isolation of toxic substances (from non-pathogenic as well as from pathogenic bacteria).

The purpose of this report is to induce uniformity in the employment of methods in the study and description of species of bacteria, and to this end it is necessary not only to recommend the routine employment of various media, and the setting forth in due order of information with reference to the morphological and biological features presented by any species that has been studied, but also to describe with exactitude the usages which the committee have deemed the most acceptable in connection with the various tests.

It is in no sense intended that these recommendations should form a complete treatise upon bacteriological technique. Only such matters are treated concerning which it is felt that there is need of greater uniformity of procedure or a more precise and correct technique. Especially in the preparation of media is greater uniformity of procedure necessary.

The committee feel that these recommendations are in many respects imperfect, yet they make them in the belief that, while in no sense final, they constitute a step towards a universally accepted method of procedure in connection with species differentiation.

SOURCE AND HABITAT.

Concerning these important facts, it need only be stated that a possible seasonal distribution of species must always be borne in mind. The date recorded on the analysis table should bear upon this fact.

MORPHOLOGICAL CHARACTERS.

1. *Form.*

To insure uniformity of description of the three main divisions and their subdivisions as determined by their grouping, the following nomenclature is adopted and the terms defined:

1. Coccus or micrococcus.
   Forms which are spherical or subspherical.
2. Bacillus.
   Oblong or cylindrical forms, having one dimension definitely greater than any other, more or less straight and never forming spirals.

3. Spirillum.

Cylindrical and curved forms, constituting complete spirals or portions of spirals.

The natural grouping which may be observed in hanging drop cultures and frequently also in cover-glass preparations, leads naturally to subdivisions of the main groups, as follows:

1. Coccus.

a. Single coccus, grouped irregularly.

b. Diplococcus, forming pairs.

c. Streptococcus, forming chains, often showing paired cocci.

d. Tetracoccus, forming fours by division through two planes of space.

e. Sarcina, forming packets of eight members, by division through three planes of space.

2. Bacillus.

a. Single bacillus.

b. Diplo- and Strepto-bacillus, forming twos or longer chains, the bacilli attached end to end.

c. Filaments, or thread-like growths, in which divisions into bacilli of the normal length are not apparent, or occur irregularly and transversely to the long axis of the growth.

The determination of morphological characters should always be made from fully developed cultures; those which are too young may present immature forms, due to rapid multiplication, while in old cultures, altered or degenerated forms may be observed.

When growth is obtained upon different media, variations, especially in size, may sometimes be observed. These differences should always be described, together with a note of the media upon which they were developed, and a statement as to whether such variation is a marked feature of the species under consideration.

The conditions of temperature and of medium which favor growth are very various for different species, so that no fixed temperature, medium, or age of growth can be determined upon as applicable to all species. Morphological descriptions should always be accompanied by a definite statement of the age of the growth, the medium from which it was obtained, and the temperature at which it was developed.

It is further advisable that the appearances observed in growths developed upon a solid and in a liquid medium, should be recorded.

In accordance with these facts, the following procedure is recommended as a routine whenever it is applicable, for the determination of the form and grouping of bacteria.

Determine and describe the morphology from growth obtained upon at least one solid medium and in at least one liquid medium. Growth at 36°-38° C. should, in general, be not older than from 24 to 48 hours,

while growth at room temperature (18° C.-22° C.) should be not older than from 48 to 72 hours. Growth on solid media may be studied from cover-glass preparations; while in liquid media growth is best observed in hanging drop, preferably in a fresh medium inoculated with a very small amount of the culture to be examined.

It is desirable as a routine procedure in recording form and grouping, that growth from nutrient broth, gelatine and agar be microscopically examined and described, and that any variation from the morphology thus established, found upon examination of growth from other standard media, be accurately noted.

2. *Dimensions.*

It seems probable that the remarkable diversity in published statements with regard to the dimensions of many of the commonest of the bacteria, is due largely to the different methods employed of preparing, fixing, and staining the cover-glass preparations.

Spreading the film:

The films should be made from a very dilute emulsion of the prescribed culture in distilled water, spread thinly upon a perfectly clean cover-glass,[1] and dried rapidly in the air.

Fixing the film:

For ordinary purposes of observation, fixing may be effected by passing the preparation three times through a flame, but for specimens to be used for accurate measurements this method of fixing is crude. To establish a uniform procedure, and to avoid distortions from overheating, it is recommended that the film be fixed by heating in an automatically regulated air bath for twenty minutes, at a temperature of 115° C. Care must be taken to keep the cover-glass from direct contact with the metal shelf of the oven, and in close proximity to the bulb of the thermometer.

Staining:

In regard to the dyes that should be used, there is some diversity of opinion among the members of the Committee. All agree that, when possible, dyes should be used cold, and that a dilute watery solution of fuchsin, made by adding 5 c. c. of a saturated alcoholic solution of fuchsin to 95 c. c. of distilled water, has a wide application. A dilute watery solution of methylene blue and one of gentian violet, made in the way recommended for preparing the watery solution of fuchsin, also are useful, as is likewise Loeffler's alkaline solution of methylene blue. These dyes, therefore, as thus prepared, are recommended by the Committee. It is necessary that all dyes be freshly prepared, as the presence of the alcohol, which rapidly evaporates, has a distinct effect in their staining properties.

[1]Cover-glasses, fermentation tubes, etc., may be freed from all organic matter adhering to them by boiling them for an hour in the following solution:

Potassium bichromate 6 parts, thoroughly dissolved in 100 parts of water, to which is then slowly added 6 parts of C. P. sulphuric acid. After boiling, the bichromate solution is allowed to cool, and is removed from the glassware by repeated rinsings in water. Cover-glasses thus cleansed are to be stored in strong alcohol.

Mounting:

Mounting in media of different refractive indices makes differences in the pictures obtained. The materials most commonly used for this purpose are water, and balsam dissolved in xylol, or cedar oil; the former having a low index of refraction, and the latter a high index. When examined in water the preparation may be ringed with vaseline to prevent evaporation and the consequent distortion resulting from partial drying. Balsam is much to be preferred, however, for general observation and for measurements of the bacteria, although for certain specific purposes other media having a higher or lower refractive index are more serviceable. Descriptions should always be made from examinations under a magnifying power not less than that given by a 1-12 homogeneous immersion lens and a No. 3 Huygenian eye-piece.

Measurement:

The most accurate method of determining the exact size of bacteria is by photography, but as photo-micrography at 1,000 diameters requires much special apparatus, the Committee feel that this mode of measurement cannot be required of all observers, although it is to be preferred where it is practicable.

Dimensions, especially transverse dimensions, should always be given in terms of the micro-millimeter (μ), and if not determined by photography, should be by as accurate measurement as can be obtained by an eye-piece micrometer. Welch recommends as a ready method for record, comparing the size of the bacteria with the diameter of the human red blood corpuscle. This is easily done by obtaining a drop of blood from the finger and mixing a portion of the diluted culture with it, a method which insures considerable uniformity and gives information as to the size of the organism in its living condition.

3. *Methods of grouping and arrangement in the growths.*

Grouping can be accurately determined from growths in liquid media and preferably in "hanging drop" cultures prepared by placing a drop of fresh medium upon a sterilized cover-glass and inoculating the edge with a minute portion of the culture, so that the growth and spread of the organisms may be watched.

This method of observation yields in general more accurate information than that obtained from "impression preparations" of surface colonies, though these latter are of distinct use, and are included among the generally useful tests.

4. *Staining powers.*

Little is definitely known about the intimate structure of the bacterial cell. Bacteria show, however, marked differences in their staining qualities, some taking the dye readily and staining uniformly, while others are more or less difficult to stain, and some show unstained or slightly stained

portions. These differences may be more or less constant, and they appear, to some extent at least, to depend upon the dye or the solution used for staining.

To insure uniformity, it is preferable to use simple aqueous solutions of the basic anilin dyes (See p. 64) and when these fail to give satisfactory results, Loeffler's methylene blue[1] is to be recommended.[2]

The intensity of staining often differs with the dye or solution employed, and it is well to study the appearances both in faintly and in deeply stained specimens.

As the action of the decolorizing agents upon stained bacteria has been insufficiently studied, much information might be gained by research in this direction. The method of staining known as Gram's method[3] is the most useful of the differential methods of staining and the Committee recommend that it should be applied to all species studied

5. *Capsules.*

All bacteria are believed to have an envelope, slimy or gelatinous in character, which causes them to adhere together and to other objects. In some cases this gelatinous zone, either from its extent or other characters, may be readily demonstrable, and to this the name "capsule" was given by Friedlaender; but in the majority of species it is very limited in extent or difficult of demonstration, so as to be visible only under special conditions. Many pathogenic bacteria present readily demonstrable capsules only in the animal body.

The term "capsulated" as applied to species of bacteria has been generally confined to those upon which this zone can, by one or other method, be seen. When found in nature, growing in moist places, or frequently in the animal body, capsules may not infrequently be observed, but when bacteria are cultivated artificially the capsule often seems to disappear entirely, even from the species which develop it in the animal body or under natural conditions, and only occasionally is the capsule preserved for a few transplantings in cultures in milk and blood serum, and in surface growths on moist agar.

The appearance of clear, unstained zones, more or less regular in contour, surrounding the bacteria, may be indicative of the presence of capsules, but cannot be considered as a demonstration of their presence. This demonstration can be considered positive only when the capsules are stained differentially from the contained organism and the tissue or other matter surrounding them.

Sometimes a differential stain is obtained by the ordinary staining methods, using the watery dyes, anilin-water gentian-violet, or normal or

[1] Mittheilungen aus dem Kaiserlichen Gesundheitsamte, Berlin, 1884, p. 439.
[2] Dyes should be freshly prepared.
[3] Fortschritte der Med. Bd. II, No. 6, 1884.

dilute carbolic fuchsin, the capsule taking on a paler shade of color than the contained protoplasm. In many other instances, however, no stain of the capsule is thus obtained, and for purposes of demonstration it then becomes necessary to resort to special staining methods to render the capsule visible. None of the methods yet devised are universally applicable to this end. Welch has found that many of the more delicate capsules may be dissolved or rendered invisible by contact with water, whether before, during, or after, the application of the dye. Many capsules are fixed by glacial acetic acid, and for this group of capsulated bacteria Welch's method of staining the capsules is most satisfactory. This method is as follows: The cover-slip specimens, prepared without water, are treated first with glacial acetic acid, which is at once allowed to drain off and is replaced (without washing in water) with anilin-oil gentian-violet solution, which is allowed to run off and is repeatedly added to the surface of the cover-glass until the acid has been displaced. The specimen is now briskly washed with a one to two per cent. solution of common salt. The specimen is to be examined in the salt solution. The proper strength of the salt solution to be used varies in different cases, sometimes the ordinary physiological solution sufficing; at other times over two per cent. may be required.[1]

In noting the presence or absence of capsules, the methods employed for demonstrating them and the conditions under which they have been observed should be fully stated.

6. *Flagella. Motility.*

So far as known, the movements of bacteria are produced only through the agency of flagella and when true motility of any species, as distinguished from Brownian movement, is observed, the presence of flagella may, from this fact alone, be assumed; although, the converse is by no means always true. Different degrees of motility may be observed at different ages of growth, young cultures showing much more active movements than older cultures, while in quite old cultures motion may be entirely suspended. Motion may be apparent in specimens from cultures of the colon bacillus eight or ten hours old, and absent from cultures twenty-four hours old. Other causes not well understood seem to influence motility, and an observation has been made by Theobald Smith that a specimen of the B. coli communis which was usually non-motile in young fluid cultures, was motile in hanging-drop preparations, when made from young cultures developed on solid media. Although species may be found which appear motionless under all conditions of growth, but which are otherwise undistinguishable from motile forms,[2] it may be stated that

1 Welch. The "Bulletin of The Johns Hopkins Hospital," December, 1892, p. 128.

2 Dr. Veranus A. Moore, in 1891, isolated from the organs of a pig a non-motile bacillus which proved to be similar in its pathogenic and cultural characters to the (motile) bacillus of hog cholera. In spite of the absence of motility, Dr. Theobald Smith determined that he could not do otherwise than place this organism in the hog cholera group, this being the solitary mark of distinction.

V. A. Moore—The nature of the flagella, etc. Journ. Am. Pub. Health Assoc Vol. 20, 1895, p. 436.

generally speaking the presence or absence of motility is a valuable test for species differentiation.

The study of motility is best made in hanging-drop preparations in bouillon prepared from young cultures as before described (p. 65), grown at or near the optimum temperature for only a few (6 to 18) hours.

For most bacteria the flagella are so fine and delicate that they are invisible in any mounting medium and under any of the magnifying powers now obtainable, except when especially stained.

Considerable practice is usually required for the successful preparation of specimens by any of the staining methods yet published; and while no single method is applicable to all species, any or all of these methods are liable to prove uncertain, even in the hands of skilled workers.

From some of the earlier studies on the staining of flagella, it seemed probable that the length, number and arrangement of these organs of motility might be so constant as to give important morphological data for species differentiation,[1] but their length has been found to vary so greatly and their number and arrangement are so inconstant, that less help can be derived from this source than was at first hoped for. It is, however, important to determine the arrangement and number of the flagella, to describe which the terms monotricha, lophotricha, amphitricha, and peritricha, introduced by Mesea,[2] are serviceable.

The methods of staining the flagella, most to be recommended, are two, one devised by Loeffler and described in Cent. f. Bakt. and Parasit., Bd. VII, 1890, p. 625, and the other by Van Ermengem, published in the Travaux du Lab. d' hygiene et de bact. de Gand. T. 1, p. 3, of which an abstract is to be found in Cent. f. Bakt. & Parasit., Bd. 15, 1894, p. 969.

Other methods, mostly modifications of Loeffler's, sometimes prove useful, among which may be recommended that of Bunge (Fortschritt der Med. Bd. XII, 1894, pp. 462-653) and that of Nicolle & Morax (Annales Inst. Pasteur, T. VII, 1893, p. 554).

In describing motility, the kind of motion observed should always be noted, whether active or slow, direct or rotary, vibratory, etc. When flagella are stained, the culture from which the preparation is made should be fully described and the method of staining noted.

7. *Spores.*

The vegetative form of the bacteria, which we have been considering, is the most usual stage of growth observed. While in this stage the bacteria, multiplying by fission, and fulfilling their various life functions, are

[1] Mesea. Revista d' igiene e sanita publica. No. 14, 1889, p. 513.
Luksch. Cent. f. Bakt. & Parasit. Bd. XII, 1892, s. 427.

[2] V. A. Moore. Wilder Quarter Century Book, p. 339.
V. A. Moore. Journal Am. Pub. H. Assoc., Vol. 20, Oct. 1895, p. 441.
Stoecklin. Annales Suisses des Sciences Medicals, I Serie, Livreson, 6, 1894.

sensitive to external influences, and may be easily destroyed by conditions which are inimical to them. Some species have the power to develop bodies very much less susceptible to deleterious surroundings, known as spores, which, under conditions favorable to their germination, develop into the vegetative forms of the organism from which they are derived. The spore does not multiply, and from the time of its formation seems to be and to remain in an absolutely quiescent or resting stage until it germinates. The function of the spore seems to be solely the preservation of the species; and it is generally believed that spores form only when growth conditions are in one or more ways unfavorable.

It is well known that spores develop within the bodies of many bacteria (endospores) and it is inferred by some that "joint-spores" (arthrospores) may develop in many other species. The former have been accurately studied, the latter have not, and indeed the whole conception of arthrospores for bacteria is rejected by most recent writers. So far as the endospores are concerned, they may be seen within the bodies of the bacteria, situated centrally or nearer the ends, and appear as bright, highly refractive bodies, which do not stain by exposure for a few minutes to the watery dyes. It should be noted whether the spores produce a swelling of the bacterial cell at the site of their formation, giving rise to clostridium and drumstick shapes. Spores may sometimes be stained with comparative ease, by the use of a hot dye, but some one of the special methods devised for this purpose must usually be resorted to.

The methods here recommended are those devised by Hauser (Münch. Med. Wochenscrift, 1887, No. 34), by Moeller (Cent. für Bakt. & Parasit, Bd. X, p. 273), and by Abbott (Principles of Bacteriology, 3d Ed., p. 146).

No single method seems applicable to the staining of all spores, however, and in many bacteria small, bright, and shiny areas which usually do not take up the dye, may often be observed. In some cases it is known that these appearances are due to the formation or deposit within the organism of vacuoles, fat or crystals, but as the differentiation of these bodies from spores is not always possible, either by optical or by staining methods, some other means of identifying spores is necessary.

It is obvious that the only means of positively knowing that a certain body is a spore, is to see it perform the only active function it can perform, namely, to observe its development into a vegetative form of bacterium. Such a study is greatly facilitated by a special incubator fitted to receive the microscope, and requires patient watching for perhaps many hours, so that although it is often possible to prove the identity of spores in this way, it is seldom a practical thing to do. The method most in vogue for this determination is to test the resistance of the suspected bodies to heat (moist heat between 80° and 100° C.). This method, as is acknowledged, is faulty, for although bodies which will withstand such a temperature for any considerable length of time must be spores, yet it is

readily conceivable that spores may also be formed which have not the power of resisting such high degrees of heat, or may be capable of withstanding only drying or possibly also other conditions which are usually inimical to bacterial life.

This method, however, is the most practical one at present at our command for the determination of the presence or absence of spores, and while the Committee urge that the germination of spores should be studied whenever possible, yet they would recommend that the present test for the presence of spores be

(1). The development of colonies of the species under examination from cultures which have been subjected to a temperature of 80° C. for ten minutes; and also,

(2). The presence of highly refracting bodies within the bacteria in unstained specimens and their demonstration as "spore-like" bodies by one or more of the approved special methods for staining spores.

8. *Pleomorphism.*

A separate section is provided for the consideration of the tendency of the bacteria to pleomorphism, a subject already referred to, because this matter in the past has scarcely received the attention it deserves.

The well known discrepancies in the descriptions of what is evidently one and the same species, given by different and equally competent observers, would appear to be largely due to a lack of recognition of this tendency; and while careful study will undoubtedly afford a more perfect knowledge of the limit of variation in size and shape among the members of a single species of bacteria, the determination of the extent of pleomorphism promises to be of definite value as an additional character to be made use of in grouping allied species.

Attention is called to the variations in size and shape brought about by the following conditions of growth:

(*a*) at different temperatures;
(*b*) upon or in media of different composition;
(*c*) upon or in media of different degrees of acidity and alkalinity;
(*d*) in cultures of different ages,
(*e*) as well as to the variations in the size and shape of different individual bacteria obtained from one culture, and appearing often in the same field of view: *i. e.*, subjected to exactly the same conditions of growth.

9. *Involution and degeneration forms.*

Little need be said in this section beyond recommending that a note be made of the changes which occur in the older cultures in the form of the bacteria, and of the period at which, under various conditions, such abnormal or irregular forms make their appearance. Up to the present

time there has been little systematic study of the modifications in the shape and appearance of bacteria in older cultures.

BIOLOGICAL CHARACTERS.

A. Cultural Characteristics.

The biological characters shown by the bacteria depend so largely upon the composition of the media upon or in which these organisms are grown, that the Committee urge most strongly the adoption by all workers in this line of standard media for use in species description and differentiation.

It is freely conceded that no two batches of media can be made absolutely identical, but much greater uniformity in the composition of media may be obtained than that which now prevails, by adhering closely to a uniform method of procedure in their manufacture.

To this end the Committee have drawn up exact methods of preparing culture media, standardized by what have proved, in their experience, the best procedures. These methods do not represent as a whole, the procedures followed by any one of their number up to the present time, and it is not claimed for them that they are in any sense perfect, but it is believed that by the adoption of standard methods of preparing media much of the divergence that now exists in the descriptions of bacteria, both well and little known, will be obviated.

The preparation of artificial culture media in general.

It is recommended that the following ingredients be uniformly employed in the preparation of the respective standard culture media:

1. Distilled water.
2. Fresh lean meat (beef, or when veal or chicken is substituted, this change should be stated).
3. Witte's pepton (dry, made from meat).
4. Sodium chlorid, C. P.
5. Sodium hydroxid, C. P., in normal solution for alkalinization.
6. Hydric chlorid, C. P., in normal solution for acidification.
7. Pure redistilled glycerin.
8. Carbohydrates, as nearly chemically pure as possible.
9. Commercial sheet gelatin, washed as free as possible from acids and other impurities.
10. Commercial agar in threads (high grade).
11. Such chemicals as are employed for special purposes, to be as pure as practicable.

Sterilization of media:

Sterilization may be effected either by the continuous or by the fractional method.

I. When sterilizing by the continuous method, some form of autoclave is to be preferred, and in using this apparatus it is requisite that the confined air be replaced by superheated steam. To insure this, both the manometer and the thermometer should be made use of, and the time decided upon for sterilization should begin only when the theoretical temperature, as indicated by the pressure gauge, corresponds with that recorded by the thermometer. Exposure in the autoclave to a temperature of 110° C. (6 lbs. pressure) for fifteen minutes is usually sufficient for the sterilization of glassware, apparatus, and media in tubes; for the sterilization of media in bulk at this temperature about thirty minutes' heating is necessary.

II. Sterilization by the fractional method may be effected either, *a*, in streaming steam, or *b*, in an incubator or water bath at some temperature not less than 60° C.

These methods are so well known as to require no description here.

Reaction of media:

The importance of the reaction of media, as a controlling factor in the development of biological characters, is well known to be very great.

The first thing to obtain is a standard "indicator" which will give uniform results. These requirements are best fulfilled by phenolphthalein.

This indicator was first suggested by Schultze in combination with the titration method for obtaining the desired reaction for culture media (Cent. für Bakt. & Parasit Bd. X, 1891, p. 53), but its general adoption seems to have been retarded largely by Dahmen (Cent. für Bakt. & Parasit, Bd. XII, 1892, p. 620) who claimed that its use was not feasible, owing to complications which might arise from the presence of carbonates and ammonium salts in the solution to be tested. These objections to the use of phenolphthalein do exist, but may be readily overcome.

The amount of free and combined ammonia present in culture media at the time the reaction is determined, has been found not to exceed 0.003 per cent., which is less than one tenth the amount which interferes with the accuracy of this indicator,[1] while the production of carbon dioxid is obviated to a very great degree by neutralizing with sodium hydroxid instead of with sodium carbonate, and any of this gas which may be absorbed from the atmosphere is practically all driven off by heat during the preparation of the media.

The great advantage in the use of phenolphthalein over other indicators lies in the fact that it takes into account the reaction of weak organic acids and of organic compounds which have an amphoteric reaction, but in which the acid character predominates. Turmeric possesses the same properties, but the change in color from a yellow to brown is less satisfactory than the development of purple-red color, and furthermore turmeric paper changes color rather slowly, while with phenolphthalein the color appears almost instantly.

[1] Ammonia is not produced by the addition of alkali to the nitrogenous compounds, because at no time during the preparation of the media is there an appreciable amount of free alkali present.

Another advantage to be gained from the use of this latter indicator, is its behavior towards the phosphates. Petri & Maassen (Arbeiten aus dem K. Gesundheitsamte, Bd. VIII, 1893, p. 311) and Timpe (Cent. für Bakt. & Parasit. Bd. XIV, 1893, p. 845; Bd. XV, 1894, pp. 394-664; Bd. XVII, 1893, p. 416) have shown that the amphoteric reaction of media is associated with the presence of phosphates, and that there are present in peptone and gelatin proteid bodies which possess both an acid and a basic nature, but in which the acid character predominates. These observers agree that to determine accurately the reaction of such amphoteric compounds phenolphthalein (or turmeric paper) should be used as an indicator.

It is known that at the neutral point of phenolphthalein any free phosphoric acid present enters into combination, and the monobasic and tribasic salts of this acid are changed to the dibasic form (Na_2 H P O_4). Now disodium hydrogen phosphate reacts alkaline to litmus, lacmoid, rosolic acid, and methyl orange, but neutral to phenolphthalein and turmeric.

Studies made at the Lawrence Experiment Station show that this acid salt may be added to culture media in amounts greatly exceeding those naturally present in the media without producing any apparent influence upon bacterial development.

From these facts it seems clear that the use of any of the above mentioned indicators, other than phenolphthalein and turmeric, in the presence of this dibasic phosphate, prevents the addition of a sufficient amount of free alkali to effect neutralization, and as the amount of phosphates in media varies considerably, the reaction passes beyond accurate control when litmus and other substances of its class are used as indicators.

Datum point to which all degrees of reaction shall be referred:

From the available evidence it seems advisable to adopt the phenolphthalein neutral point as the fixed point to which all degrees of reaction shall be referred.

The question of the proper reaction of media for the cultivation of bacteria and the method of obtaining this reaction, have been discussed in a valuable paper by Mr. George W. Fuller, published in the Journal of the American Public Health Association, Vol. 20, October, 1895, p. 321. Some of the main results there given have been mentioned above.

Method of determining the degree of reaction of culture media:

For this most important part in the preparation of culture media, burettes, graduated into one tenth c. c., and three solutions are required.

1. A 0.5 per cent. solution of commercial phenolphthalein in 50 per cent. alcohol.

2. A $\frac{n}{20}$ solution of sodium hydroxid.

3. A $\frac{n}{20}$ solution of hydric chlorid.

Solutions Nos. 2 and 3 must be accurately made up and must correspond with the normal solutions soon to be referred to. Solutions of sodium hydroxid are prone to deterioration from the absorption of carbon dioxid and the consequent formation of sodium carbonate. To prevent as much as possible this change, it is well to place in the bottle containing the stock solution a small amount of calcium hydroxid, while the air entering the burettes or the supply bottles should be made to pass through a "U" tube containing caustic soda, to extract from it the carbon dioxid.

The medium to be tested, all ingredients being dissolved, is brought to the prescribed volume by the addition of distilled water to replace that lost by boiling, and after being thoroughly stirred, five c. c. are transferred to a 6-inch porcelain evaporating dish; to this forty-five c. c. of distilled water are added, and the fifty c. c. of fluid are boiled for three minutes over a flame. One c. c. of the solution of phenolphthalein (No. 1) is then added and by titration with the required reagent (No. 2 or 3) the reaction is determined. In the majority of instances the reaction will be found to be acid so that the $\frac{n}{20}$ sodium hydroxid is the reagent most frequently required. This determination should be made not less than three times, and the average of the results obtained taken as the degree of reaction.

One of the most difficult things to determine in this process is exactly when the neutral point is reached as shown by the color developed, and to be able in every instance to obtain the same shade of color. To aid in this regard, it may here be remarked, that in bright daylight the first change that can be seen on the addition of alkali is a very faint darkening of the fluid, which on the addition of more alkali becomes a more evident color, and develops into what may be described as an Italian pink. A still further addition of alkali suddenly develops a clear and bright pink color, and this is the reaction always to be obtained.

All titrations should be made quickly and in the hot solutions, to avoid complications arising from the presence of carbon dioxid.

When this manipulation is carried out uniformly, as here suggested, and the end point having the same intensity of color is always reached, very satisfactory and closely-agreeing results may be obtained.

Neutralization of media:

The next step in the process is to add to the bulk of the medium the calculated amount of reagent, either alkali or acid as may be determined. For the purpose of neutralization a normal solution of sodium hydroxid or of hydric chlorid is used and after being thoroughly stirred the fluid thus neutralized is again tested in the same manner as at first to insure the proper reaction of the medium being attained. When neutralization is to be effected by the addition of alkali, it not infrequently happens that after the calculated amount of normal solution of sodium hydroxid has

been added the second test by titration will show that the medium is still acid to phenolphthalein, to the extent sometimes of from 0.5 to 1 per cent. This discrepancy is perhaps due to side reactions which are not understood; the reaction of the medium, however, must be brought to the desired point by the further addition of sodium hydroxid, and the titrations and additions of alkali must be repeated until the medium has the desired reaction, (*i. e.*, 0.0 per cent.—0.005 per cent., see below).

After the prescribed period of heating, it is frequently found that the medium is again slightly acid, usually about 0.5 per cent. Without correcting this the fluid is to be filtered and the calculated amount of acid or alkali is to be added to change the reaction to the one desired.

A still further change in reaction is not infrequently to be observed after sterilization, the degree of acidity varying apparently with the composition of the media and the degree and continuance of the heat.

Manner of expressing the degree of reaction of culture media:

Since at the time the reaction is first determined culture media are more often acid than alkaline, it is proposed that acid media be designated by the plus sign and alkaline media by the minus sign, and that the degree of acidity or alkalinity be noted in parts per hundred; thus a medium marked +1.5 would indicate that the medium was acid and that 1.5 per cent. of $\frac{n}{1}$ sodium hydroxid is required to make it neutral to phenolphthalein, while—1.5 would indicate that the medium was alkaline and that 1.5 per cent. of $\frac{n}{1}$ acid must be added to make it neutral to the indicator.

Limits of accuracy of the proposed method for the control of the reaction of media:

The available data are as yet insufficient to warrant any conclusions upon this point. The limits of accuracy seem to vary with the ingredients employed in preparing nutrient media, different samples of meat infusion, pepton, and gelatin appearing to react differently with the acids and alkalis and in a way which is not understood.

This method, nevertheless, when carefully carried out, and when the media before titration are thoroughly mixed and are of the prescribed volume, gives fairly uniform results.

Standard reaction of media (provisional):

Experience seems to vary somewhat as to the optimum degree of reaction which shall be uniformly adopted in the preparation of standard culture media. To what extent this is due to variation in natural conditions as compared with variations of laboratory procedure, it seems impossible to state. Somewhat different degrees of reaction for optimum growth are required, not only in or upon the media of different composition and by bacteria of different species, but also by bacteria of the same species when in different stages of vitality.

The bulk of available evidence from both Europe and America points to a reaction of +1.5 as the optimum degree of reaction for bacterial development in inoculated culture media; and while this experience is at variance with that in several of our own laboratories, it has been deemed wisest to adopt +1.5 as the provisional standard reaction of media, but with the recommendation that the optimum growth reaction be always recorded in species descriptions.

The preservation of culture media in stock:

From what has been said, it is evident that species differentiation can be best determined upon media made in a single batch, and it is therefore advisable to preserve a special set of media for this purpose. Such stock, however, is useful only when properly preserved, and to this end contamination and drying must be particularly guarded against.

The following methods of preserving media are suggested:

For preserving media in bulk the method employed at the Pasteur Institute in Paris is most efficient. Bulbs of moderately thick glass, with a capacity of from ½ to 2 liters, and provided with a long, narrow neck, must be secured.

The neck of the flask is first drawn out in the flame of a blow-pipe, two or three cm. above the bulb; the flask is then filled up to the neck with the desired medium; the opening is plugged with cotton and sterilized in an autoclave. After sterilization the flask is hermetically sealed in a blow-pipe flame at the point of constriction.

Other very efficient and perhaps more practical methods are, after sterilization of the media in ordinary flasks, to cut off the cotton plug with a hot scissors, singe the free end of the plug in a flame, and cover the top of the tube or flask with thin sterilized tin foil[1] which may be held in place by a small rubber band, or sealed with paraffin; or, seal with paraffin either by dipping the engaged end of the cotton plug in paraffin previously sterilized at 150° C., and replacing it while hot in the tube or flask, or cover the free end of the cotton plug with paraffin which melts at a temperature not lower than 40° C.

Stored media should always be kept in a dark place, and preferably at a low temperature.

The preparation and use of artificial culture media in particular:

As the exact methods employed by different workers in bacteriology in preparing culture media used for species differentiation, are so seldom described, and as the majority of published articles dealing with descriptions of new species make little or no mention of the mode of preparation of the commonest, and therefore the most important, culture media, the Committee have deemed it necessary to draw particular attention to this fact and to devise a standard procedure to be followed in the preparation

[1] Tin foil may be sterilized by storing sheets of the desired size in 0.5 per cent. solution of carbolic acid. These should be rinsed in sterilized water, or passed quickly through a flame immediately before use.

of such media as are here described, when used for species description and differentiation.

1. *Nutrient broth.*

One part of finely chopped, fresh, lean meat is macerated in two parts of distilled water in an ice-chest for from 18 to 24 hours, stirring the mixture occasionally. The infusion is strained while cold through a fine cloth, and to the clear filtrate 1 per cent. of pepton and 0.5 of sodium chlorid are added.[1] The mixture is then heated over a water-bath until all the added ingredients are dissolved, when by the titration method (see p. 75) a sufficient amount of normal solution of sodium hydroxid is added to make the whole solution feebly alkaline (practically neutral) to phenolphthalein.

The medium is then heated over a water-bath for 30 minutes and boiled for five minutes over a free flame. While still hot it is filtered either through filter paper, or through a fine cloth and absorbent cotton, and to the filtrate sufficient $\frac{n}{1}$ hydric chlorid is added, the amount being determined by the titration method, to give the medium the desired reaction (+ 1.5). If the medium is perfectly clear it is now distributed in tubes and flasks and sterilized (see p. 76).

Should the medium prove not to be clear after filtration, it must be cleared. This is best done by mixing with each 1 liter of the medium, cooled to 50° — 60° C, the whites of one or two eggs stirred in a little of the medium, and boiling vigorously. The hot fluid is then again filtered and when clear is distributed and sterilized.[2]

The use of nutrient broth for species differentiation:

For the purpose of obtaining a more uniform description of the characters developed by bacterial growth in nutrient broth in test tubes, attention must be given to the liquid itself, and the appearances on its surface and at the bottom of the tube.[3]

The special appearances to which attention is drawn are given in some detail in the chart or analysis table accompanying this report, and the effect upon the cultures of the reaction and composition of the medium, and the temperature at which growth develops are referred to elsewhere.

Preparation of fermentation broth:

Fermentation broth is usually prepared in the same manner as nutrient broth except that 1 per cent. of sugar is added either at the time of adding the pepton or to the completed nutrient broth. It is important that the broth to which the sugar is to be added should be free

[1] Theobald Smith states that if pepton is added before a preliminary boiling most of it is lost for diphtheria toxin; he therefore recommends that the clear filtrate obtained by straining be boiled and filtered and that the solid ingredients be added and dissolved thereafter. See Trans. Assoc. of American Physicians, 1896. On appearance of toxin in cultures of diphtheria.

[2] Nutrient broth may be made in considerable quantity and kept in stock, as it serves as a basis for other nutrient media. (See pp. 79–80.)

[3] See Th. Smith, "The relative value of cultures in liquid and solid media in the diagnosis of bacteria." Botanical Gazette, Vol. XI, No. XI, and Medical News, 1886, Nov. 20, p. 57.

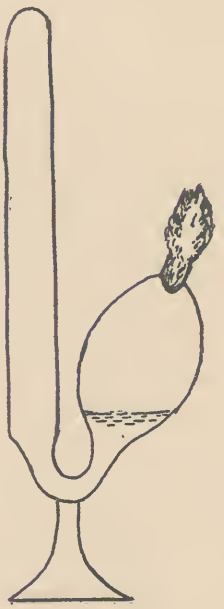
from sugar. Ordinary meat broth is likely to contain some muscle sugar.[1]

The sugars recommended for the fermentation test are glucose, lactose, and saccharose, and these give the reaction most frequently in the order named.

Sterilization of media containing sugars is best effected in streaming steam at 100° C. on each of three consecutive days, for the reason that changes in the sugars may occur at the higher temperatures obtained in an autoclave.

For the purposes of this test a tube of special construction is required[2] which is commonly known as the "Fermentation tube."

This is essentially a tube 1.5 c. m. in diameter bent at an acute angle, closed at one end and provided with a bulb at the other, which latter should be large enough to receive all the liquid contained in the closed branch, should gas in any considerable quantity form and collect there.[3]



This tube also serves a most important end in giving information as to the aerobic and anaerobic growth of the species under examination, for the connecting tube, being constricted, serves to prevent to a great degree, the entrance of the oxygen of the air into the closed branch, and the free oxygen[4] in the medium is driven out by heat during the process of sterilization; from which it may be seen that growth in the bulb, to which access of oxygen is allowed, is aerobic, while growth in the closed branch, from which oxygen is excluded, is anaerobic.

For method of cleansing the fermentation tube see p. 64.

For the study of fermentation alone, ordinary tubes of 1.5 cm. calibre sealed at one end and bent to an acute angle will serve. They do not answer so well, however, for observations upon the anaerobic properties of bacteria, the connecting tube being too broad.

[1] The Journal of Experimental Medicine, Vol. II, No. 5, p. 546, recommends the following simple method of preparing dextrose-free bouillon: "Beef infusion, prepared either by extracting in the cold or at 60 degrees C., is inoculated in the evening with a rich fluid culture of some acid-producing bacterium (I use temporarily B. coli) and placed in the thermostat. Early next morning the infusion, covered with a thin layer of broth, is boiled, filtered, pepton and salt added and the neutralization and sterilization carried on as usual. Such bouillon when tested with B. coli in the fermentation tube will no longer permit any anaerobic growth. The closed branch remains clear, a sign that carbohydrates are absent." Bouillon prepared in this way does not contain indol or nitrites. Spronck's method of allowing beef to decompose for several days, is unreliable because bouillon made from it frequently contains relatively much sugar (Th. Smith).

[2] See Theobald Smith, Wilder Quarter Century Book, Ithaca, 1893, p. 187.

[3] Made by Emil Greiner, 146 William street, New York City.

[4] The bubbles which collect in the closed branch after each heating during sterilization, should be removed by tilting the tube while the medium is still very hot, otherwise they would be again absorbed by the fluid.

2 and 3. *Gelatin plates and tubes.*

Nutrient gelatin may be made from nutrient broth in stock, by the addition to the broth of 10 per cent. of sheet gelatin and neutralizing by the process already explained. (See p. 74.)

When prepared in this way it is advisable as a routine procedure to add the whites of one or two eggs after the gelatin has been melted and the required alkali added, which when brought to a hard boil coagulates firmly, clears the medium, and allows of easy and rapid filtration.

The other procedure recommended is similar to that for the preparation of nutrient broth (see p. 77) except that with the pepton and salt, 10 per cent. of sheet gelatin is added.

The solid ingredients are heated in the meat infusion until the gelatin is melted and the pepton dissolved, and the medium is then made neutral, or faintly alkaline, by the addition of a sufficient amount of normal sodium hydroxid solution, the quantity being determined by titrating with phenolphthalein. The solution is then heated for 25 minutes in a steam- or water-bath, boiled for 5 minutes over a free flame, cleared by filtration and rendered of the standard reaction, or other desired reaction, by the addition of hydric chlorid in normal solution. The completed medium is then distributed in sterilized tubes and flasks and sterilized.

Sterilization of gelatin can be satisfactorily effected in the autoclave by a single heating for 15 minutes at 110° C, or even at 115° C.[1]

Cultural characters in and upon nutrient gelatin :

A great objection to gelatin as a component of media for the differentiation of species of bacteria lies in its great variability in reaction, melting point, etc.,[2] nevertheless, nutrient gelatin is indispensible for bacteriological work.

From what can be learned of the manufacture of gelatin, the production of an article having a definite melting point and a definite reaction presents peculiar difficulties, and therefore the Committee are unable to recommend any of the commercial sheet gelatins they have used as being always satisfactory.

All that can be recommended at present is that an acid-free gelatin be employed which in a 10 per cent. solution remains solid at 24° C. after being subjected to the required sterilization.

The characters to be recorded which develop either in the deep and surface colonies on gelatin plates or from "streaks" and "punctures" in tube cultures are referred to in the annexed analysis table.

It is most important in studying growths upon nutrient gelatin that the medium should be of uniform consistency throughout. To insure this and to prevent a greater density of the medium on the surface layers

[1] See Wyatt Johnston, Jour. Am. Pub. Health Assoc., Vol. 20, Oct. 1895, p. 482.

[2] For a discussion and record of experiments upon different forms of gelatin, see Sedgwick & Prescott, Jour. Am. Pub. Health Assoc., Vol. 20, Oct. 1895, p. 450.

through drying, the medium in tubes should be preserved as suggested (see p. 76) or, where a slight surface drying has occurred, the medium may be melted, thoroughly mixed and again solidified immediately before using.

Growths in tubes (punctures) present very different appearances in surface dried media from those observed in media freshly prepared; and in plates, either rolled or poured, it may often be observed that colonies approaching the surface do not break through and spread upon the surface when the medium is in any way dried. To prevent the medium in Petri dishes from drying, several dishes may be placed in a pile and weighted, the cotton plug of a tube culture or an Esmarch roll tube after being singed in a flame may be sealed with paraffin, or covered with sterilized paper, rubber tissue, or tin foil and bound with a thread or rubber band.

4 and 5. *Agar plates and tubes.*

Nutrient agar, like nutrient gelatin, may be made from nutrient broth already prepared and kept in stock, by melting in the broth from 1 per cent. to 2 per cent. of thread agar and changing the reaction as may be necessary after titration. With regard to the reaction after adding and dissolving the agar, it may be found unchanged or may be too alkaline, agar being either neutral or faintly alkaline in reaction. Agar may be melted in the broth in the autoclave (115° C. for 20 minutes) without loss of volume, but when for any reason the autoclave is not used the mixture should be heated over a free flame. As this boiling is necessarily somewhat prolonged there will be a considerable loss in the volume of the medium and consequent concentration, which latter may be avoided by the previous addition of 200—300 c. c. of distilled water for each 1 liter and the subsequent adjustment of the bulk of the medium before neutralizing by further evaporation if the bulk be too great or by addition of distilled water if the bulk be too small.

Nutrient agar may also be prepared according to the procedure recommended for the preparation of nutrient broth (see p. 77), with the exception that from 1 per cent. to 2 per cent. of thread agar is added with the pepton and salt. The suggestions noted above in referring to the melting of agar in nutrient broth pertain with equal force here. The steps in the procedure are as follows: To the required bulk of clear meat infusion add 1 per cent. of pepton, ½ per cent. of sodium chlorid, and from 1 per cent. to 2 per cent. of thread agar; melt and dissolve the ingredients either in an autoclave or over a free flame, in which latter case additional water should be added to the mixture. The medium is then brought to the prescribed volume, and rendered neutral or faintly alkaline and steamed in a steam sterilizer for 30 minutes. The original bulk is then restored by the addition if necessary of hot distilled water, the solution filtered through filter paper or through cloth and absorbent cotton, and the medium is then brought to the desired degree of reaction

(standard reaction = + 1.5 per cent.) by the addition of the calculated amount of normal solution of hydric chloride, after titration with phenolphthalein as an indicator.

The clear medium[1] is then distributed in sterilized flasks and tubes, and sterilized (see p. 72).

When "glycerin agar" is to be prepared, 6 per cent. by volume of pure, redistilled glycerin is added and thoroughly mixed with the hot nutrient agar just previous to its distribution in flasks and tubes, after which it is sterilized.

Agar tubes should be freshly melted and inclined just previous to use and a sufficiency of condensation water should be present, unless for some special purpose dried out agar is desired.

The technical difficulties in the way of successful "plating" in agar are considerable, and a recital of the experiences of the Committee in this particular may not be out of place. Plates may be made either by streaking the surface of an agar film cooled in a Petri dish, or by making dilutions in the usual way and pouring the infected medium into Petri dishes or by forming a film within the tube (Esmarch roll tube).[2] Streak plates require no particular directions for their preparation. Pour plates and roll tubes are best made by boiling the agar in the tubes in a water-bath for 10 minutes and then transferring them to a bath of 42° C. for 10 minutes. The inoculations and dilutions should then be made rapidly and the medium poured into Petri dishes, or rolled in a groove made in a piece of ice, or in a stream of cool water. Some practice is required for making serviceable agar roll tubes, but the method is practical. To prevent the film from slipping down, the tube should be placed in a slanting or horizontalp osition for 12–18 hours, to permit the upper thin edge of the medium to dry slightly and adhere to the glass; after this time the tubes may be placed vertically.

The spreading of growth in the condensation of water in agar plates may be largely prevented by allowing the excess of moisture to evaporate by canting the covers upon the dishes, while protected by a bell-jar, for 10–15 minutes before putting the plates in the incubator.

6. *Potato as a culture medium.*

In spite of the differences in the reaction and composition of the potato as obtained in different varieties, or from various sources or even from the same source, and used at different periods of the year, this tuber is very valuable as a culture medium.

Some species of bacteria, it is true, grow with great uniformity on potato from whatever source, but others grow so differently on potatoes of different composition that they would not be recognized as cultures of the same organism.

[1] Should the medium prove not to be clear, it must be cleared with the white of egg (see p. 77).

[2] Zeit.fur Hyg., Bd. I, 1886, p. 293.

To obtain satisfactory results two courses are open; the first, and the one which is less generally useful, is to employ the old method of boiling and sterilizing the whole potato, then cut the tuber in half and inoculate one portion with the culture under examination and the other with a control culture.[1] This method may well be employed in identifying species resembling the "B" typhosus.

The second course, which is generally the preferable one, is to correct the reaction of the potato, which will often be found to be acid. Much, if not all, of the acid may be removed from the prepared potato cubes[2] by prolonged washing (12 to 18 hours) in cold running water. A quantity of potatoes obtained at one time from the same source and prepared as suggested may be stored in mass, as proposed by Plaut.[3] These will generally be found to give quite uniform growth appearances. Should it be desired to change the reaction of the potato, this may be readily done by steaming the potato cubes in a measured quantity of distilled water for half an hour, testing the reaction of the water with phenolphthalein and adding the required amount of $\frac{n}{1}$ sodium hydroxid, after which the boiling should be repeated for thirty minutes, and the potato cubes may be then put into tubes.

Potatoes are preferably sterilized in streaming steam for 30 minutes on each of three consecutive days, or the autoclave may be used.

7. *Milk as a culture medium.*

Milk to be used as a culture medium should be as fresh as possible, and should be heated for 15 minutes in a steam sterilizer, in a cotton plugged flask, then placed in an ice-box over night to allow the cream to rise and the suspended matters to deposit. The milk is then siphoned off into a flask, free from the cream and deposit, and its reaction is tested to phenolphthalein, by titration (see p. 74). Should the milk prove to be less than 2 per cent. acid to this indicator it may be at once filled into sterilized cotton plugged test tubes holding about 20 c. c. each and sterilized for 20 minutes on each of three (preferably four) consecutive days in streaming steam. Should the milk, however, prove to be more markedly acid, it should be rejected, or rendered 1.5 per cent. acid to phenolphthalein by the addition of $\frac{n}{1}$ sodium hydroxid; it may then be filled into tubes and sterilized as above directed. A solution of litmus may be added to milk just previous to its distribution in tubes, in sufficient quantity to give the milk a pale blue color. The presence of litmus in the medium facilitates greatly the determination of its reaction.

Professor Conn has pointed out that milk is difficult to sterilize, and

[1] Germano u. Giorgio Maurea. Vergleichende Untersuchungen über den Typhusbacillus und ähuliche Bacillen. Beiträge z. pathol. Anatomie, XII, (1893). S. 494.

[2] Roux. Annales Inst. Pasteur, T. 1888, p. 28. Bolton. Med. News, Vol. 1, 1887, p. 318.

[3] Cent. f. Bakt. & Parasit. Bd. III, pp. 100–127.

recommends that after heating on each of four consecutive days the tubes be kept in an incubator at 37.5° C. for several days before they are used for culture purposes. This full procedure is recommended when practicable, although the Committee do not consider the incubation of the milk tubes before inoculation to be indispensable. One or more of the unsowed milk tubes made from the same sample should, in any event, be kept under the same conditions as the tubes containing cultures and carefully compared with them from time to time, as without such control errors are likely to occur.

The changes in milk produced by bacterial growth render this medium one of the most valuable we have for species differentiation and identification, and the Committee are greatly indebted to Professor Conn for the tables he has compiled for recording these various changes, and which are embodied in the appended analysis table.

The formation of peptons and albumoses in milk must be determined by chemical tests and of these, that known as the "biuret reaction" seems the most available. This reaction is obtained by rendering the fluid to be tested distinctly alkaline with an excess of sodium or potassium hydroxid in solution and then adding cautiously a very dilute solution of cupric sulphate when a fine, more or less deep rose color is produced; by adding an excess of cupric sulphate the rose color is changed to violet. This reaction is common to both peptons and albumoses, and when, as is probable in many cases, both these substances are present it is desirable that a differentiation between them should be made.

The several albumoses may be precipitated by reneutralizing the culture which has given the biuret reaction, making it faintly acid with a trace of acetic acid and adding ammonium sulphate to complete saturation; the precipitated albumoses are then removed by filtration; the peptons, however, are still in solution and their presence in the filtrate may be determined by again applying the biuret test, the intensity of the color reaction giving a rough indication of the proportional amount of albumoses removed by precipitation.[1]

8. *Blood serum*[2] *as a culture medium.*

Blood of the beef or sheep, collected in large, wide mouthed jars (preferably straight sided museum jars of from ½ gal. to 1 gal. capacity), previously sterilized, should be brought to rest in the laboratory as soon as practicable. Just after coagulation has commenced, usually within 20 minutes, the adhesions betwen the coagulum and the jar are to be carefully broken up with a sterilized glass rod and the jars are then placed in an ice-chest for 24 hours to allow the serum to separate from the clot. At this time one or more inches of a straw colored to reddish serum will be found upon the surface which may be readily siphoned off into steri-

[1] See Gamgee's Physiological Chemistry, Vol. II, p. 124, et seq. or other treatises on this subject.

[2] Koch, Berlin Klin. Wochens, 1882, No. 15.
Koch, Mittheil, a. d. k. Gesundheitsamte, 1884, Bd. II, s. 48.

lized cotton-plugged, Erlenmeyer flasks of about 200 c. c. capacity, or into "preserve jars" or other convenient vessels which may be tightly sealed. These vessels containing serum may be cotton plugged and returned to the refrigerator for 24–48 hours to allow of a further deposition of the coloring matters, or the serum may be preserved[1] by adding to it a little more than 1 per cent. of chloroform and sealing the flasks tightly; or the serum may be distributed in tubes and small flasks and sterilized by heat.

Blood serum may be sterilized and remain fluid, or may be rendered solid by the degree of heat used in sterilizing.

For the sterilization[2] of fluid serum it is requisite that it be exposed to a temperature of from 62° C. to 66° C. for one hour on each of six consecutive days. The best apparatus for obtaining and maintaining this temperature (about 65° C.) is a capacious and well-regulated incubator, into which the tubes and flasks containing serum are to be put each day and in which they are to be left for the prescribed time after having been warmed to the desired temperature.

Serum may be solidified[3] and still remain translucent at a temperature of 76° C., but when heated to a higher degree, a more definite coagulation takes place and the medium becomes opaque. Care must be taken in coagulating blood serum at the higher temperatures to run the temperature up slowly and not to heat above 90° C. until the serum has firmly coagulated, for, unless these precautions are taken, ebullition is likely to occur which will lead to the formation of bubbles and an unevenness of the surface upon which growth is to be obtained and studied. Serum may be solidified at the temperatures mentioned in an incubator, water oven, or hot air sterilizer, and when coagulated firmly (90° C.) the tubes and their contents may on the following day be sterilized in streaming steam at 100° C. without danger of the subsequent formation of bubbles.

Serum preserved with chloroform may be freed from the deposit which forms by filtration and then being filled into sterilized culture (test) tubes, is to be sterilized by exactly the same methods as are employed in sterilizing fresh serum. The chloroform, being volatile, tends to disappear at ordinary temperatures, but is quickly and surely driven off at the temperatures used in sterilizing.

Serum may be efficiently sterilized by passing it through a Pasteur or Berkefeld filter, under pressure. When so treated the fluid is very clear and light colored.

A very important modification of blood serum as a culture medium for

[1] M. Kirchner, Zeits f. Hyg. Bd. VIII, p. 465.

[2] Very practical points in the sterilization of blood serum may be found in Hueppe's Bakterien forschung, 1891, 5th Auflage, p. 215, etc.

[3] Tubes containing serum to be solidified should always be placed obliquely, so that the medium will present an extended surface for growth.

bacteria has been suggested by Loeffler.[1] This consists of the addition of one part of a 1 per cent. glucose broth to three parts of the fluid serum. This mixture, known as "Loeffler's blood serum," forms a very important addition to our culture media, and is to be sterilized in the same manner as blood serum, being serviceable either as a fluid or solid medium.

Serum obtained from the serous cavities of the body, whether occurring with or without inflammation, may be used in the same manner as serum obtained directly from blood. These effusions, being obtained from the human body, are believed to be more efficient for the cultivation of certain pathogenic species than are sera from blood of the lower animals. By suitable precautions they may often be obtained absolutely sterile, thus avoiding the necessity of subsequent sterilization, and their manner of use corresponds exactly to that of serum.

Some of the advantages to be obtained from the use of serum and exudates are that they offer a better medium for the growth of many bacteria than do the media compounded from meat or vegetable extracts; a number of species do not grow upon or in them at all, and further, certain species possess the power of liquefying coagulated serum, all of which facts may prove of value in the work of identification and differentiation.

B. Biochemical features.

1. *Temperature Relations.*

The main points to be determined in studying the relations of the bacteria to temperature are three:

I. The extreme limits within which development occurs.

II. The most favorable temperature for development.

III. The thermal death point for the bacteria without spores and for the spores also when they are present.

The determination of all these points is important and should, when possible, be made. For determining the extreme limits within which development may occur and the most favorable temperature for development, a number of incubators set at different temperatures are required, and as in a small laboratory such an amount of apparatus is not usually to be had, the Committee have deemed it wiser to require that only the comparative activity of growth at 18° to 22° C. and at 36° to 38° C. must be studied, this information to be coupled with such other facts as may be learned during the study of the organism bearing on this point.

The determination of the thermal death point is of such importance that it cannot be dispensed with.

In determining the thermal death point the facts required to be known are 1, the time of exposure to heat; 2, the presence or absence of

[1] See Loeffler, Mittheil Kaiserlichen Gesunderheitsamte, Bd. II, 1884, p. 461.

moisture; 3, the presence or absence of spores; 4, the age of the culture; 5, the composition and reaction of the medium in which its resistance is tested; 6, the amount of the culture used for the tests, and 7, the character of the containing vessel.

The temperature required to destroy the species under consideration is to be determined within 2° C., thus, if samples are exposed to temperatures of 50°–52°–54°–56°–58°–60° C., and it is found that development in a suitable medium occurs after exposure to 56° C., but not after exposure to 58° and 60° C., the thermal death point is to be given as 58° C., although further study might show that it was somewhat less than this.

It is necessary to limit the time of exposure in order that all organisms may be subjected to the same conditions, and to this end 10 minutes would seem from experience, to be the most suitable period to select.

In determining the thermal death point cultures should always be moist. The experiments of Koch and Wolfhügel have shown that in a desiccated condition and subjected to dry heat, bacteria show a much greater resistance to high temperatures than when moist.

The presence or absence of spores may often be determined by the observation of stained specimens, but this method of study alone is sometimes insufficient, as is evidenced by the fact that B. erythrosporus, and possibly also other species, contain spores which are obscured rather than made evident by any dye having a reddish tinge. For this determination specimens of the culture should be observed unstained, and stained with dyes of various hues as well as subjected to a temperature of 80° C. for ten minutes. (See p. 69).

In old cultures the power of resistance of many or all of the cells may be somewhat diminished. To avoid errors arising from this possible loss of resistance, it is recommended that the cultures to be tested be grown for forty-eight hours in standard nutrient broth, and when possible at 36°–38° C. Three loopfuls[1] of this culture, when thoroughly mixed by agitating, are to be transferred to tubes containing 10 c. c. of fresh broth for the purposes of the experiment.

Using a measured quantity of standard nutrient broth to heat the culture in would seem to best serve for this determination, as its reaction has been pretty accurately determined and it is known to be a fluid which is favorable to the development of the culture. Placing the measured quantity of the culture in a known quantity of the broth defines the degree of dilution, and after exposure to a given degree of heat for 10 minutes, no transference to other culture media is required, but the tube, just as it

[1] Uniformity in the size of the loop employed is of some importance, and may be obtained by following the directions here given: Take a piece of No. 27 platinum wire 2½ times the length desired for the loop and bend it through the middle over a bit of No. 10 wire. The ends are now twisted throughout their full length and the "loop" thus formed is fastened into a glass rod to serve as a handle. In measuring out a loopful of culture it is important to observe that the loop holds all the fluid it can; that is, that it forms a biconvex body and is not simply a film covering the space within the loop.

has been experimented with, may be placed under conditions favorable for the development of any of the organisms which may have survived.

The culture (test) tubes used in these determinations should be of thin glass and uniform in size, approximately 16 mm. in diameter.

A number of accurate procedures have been used to define the thermal death point of bacteria,[1] but that which from its simplicity and the employment only of ordinary laboratory apparatus particularly recommends itself, is as follows:

Heat a capacious water-bath to the desired temperature, and place in the water in immediate contact with the thermometers, the several tubes containing 10 c. c. of standard nutrient broth. After 15 minutes' exposure to this temperature, *i. e.*, when the broth in the tubes has attained the temperature of the bath as indicated by the thermometers, the broth is to be inoculated with three loopfuls of the culture to be experimented with, by simply removing the cotton plugs, not removing the tubes from the bath,[2] and after exposure to such temperature for 10 minutes the tubes are removed and placed at once in a vessel of cold (ice) water to cool them rapidly and prevent further action of the heat upon the bacteria after the expiration of the specified time of exposure. These tubes when cooled are ready for incubation, and should be placed at a temperature favorable for the development of the species experimented with; and to allow for the recovery of such cells as have been injured but not killed by exposure to these temperatures, all incubated tubes should be kept under observation for not less than seven days.

2. *Relation to free oxygen.*

Although the study of obligatory anaërobes is steadily assuming a greater importance, the Committee feel that the subject of anaërobic culture cannot properly be fully considered here; yet, as the relation of growth to free oxygen divides bacterial species into two great classes with perhaps more sharpness than any other of the distinguishing tests, it is important that this test should always be applied in the determination of characters for species differentiation and identification.

In plate cultures, the study of growth under a "mica plate" as suggested by Koch may be recommended, while for tube cultures the more intricate methods of study are to some extent supplanted by carefully observing growth in the "fermentation tube" (see p. 78). Growth indicated by cloudiness in the bulb only, is to be found among obligatory aërobes; in the closed branch only, among obligatory anaërobes, while growth occurring in both arms of the tubes indicates the faculty of growth either in the presence or absence of oxygen (facultative anaërobes).

[1] Sternberg. Proceedings of Bacteriological Congress, Journal of the American Public Health Association, etc.

[2] It is important that the culture should be placed in the broth and not left upon the side of the tube where it may dry. The culture may be mixed with the broth by slightly agitating the tubes.

3. *Relation of growth to acidity and alkalinity of media.*

Numerous investigations have shown that varying relations exist between the reaction of media and the development of different species of bacteria, but owing to the lack of uniform and accurate methods it is difficult to compare many of the results obtained by different observers one with the other.

Nevertheless, while it has been shown that most species grow best in nutrient media rendered faintly alkaline to litmus, a sufficient number of exceptions to the rule have been found, to prove that a study of the relation of growth to the reaction of the medium is an important auxiliary in species differentiation.

In the vast majority of descriptions, no mention whatever is made of this matter, and probably no attention has been given to it. For this study all that is necessary is to add to tubes containing equal quantities of any of the standard nutrient media a calculated amount of standard solution of hydric chlorid or of sodium hydroxid, to obtain the desired reaction.

The evidence is still too meager to warrant the recommendation of the uniform use of any one kind of medium for this purpose, but in view of the valuable characteristics that may be obtained it is recommended that a record be made of cultures upon at least one medium reacting + 3 per cent., + 1.5 per cent., neutral, and — 1.5 per cent. to phenolphthalein (see p. 75).

4. *Action upon gelatin and agar.*

The liquefaction of gelatin, which often produces such characteristic appearances in plate and tube cultures, seems to be brought about by the action of some enzyme or ferment produced by the bacteria.

The nature of the ferment is perhaps not as important to the bacteriologist as a proper interpretation of the effects produced, and it may seem not inappropriate here to give a few points indicating the information which may be obtained from an intelligent examination of the growths appearing on plates and in tubes.

Deep colonies in gelatin :

Colonies developed from a single bacterium entirely surrounded by the medium appear at first as minute spots, which increase gradually in size and have a high index of refraction.

At this time they are usually circular (spherical) and sharply defined, and refract light differently from the medium and frequently they appear to have a definite texture, either coarsely or finely granular. In plates crowded with colonies an arrest of growth may be observed and for this reason only colonies which are isolated are to be used for the study of development.

The spread of colonies occurs as might be supposed in the direction of least resistance, so that if growth be equally vigorous throughout, deep colonies in solid media should be spherical. Other forms of colonies are

often observed, however, due to greater activity of certain parts of the growth, perhaps to different consistence in parts of the medium, or to the development of minute gas bubbles which split the medium, and permit the spread of the growth along the surfaces of the fissure.

If the growth be near the surface and break through upon it, the appearances presented are often quite characteristic.

Surface colonies on gelatin:

Colonies wholly upon the surface present at times certain differences from those growing up from the deeper layers of medium, especially in the liquefying and chromogenic species. Color is usually produced only in the presence of oxygen, but may be developed only in the absence of oxygen as in the case of the Spirillum rubrum.

Liquefaction of the medium often gives rise to appearances which viewed under low powers of the microscope, are very characteristic; and the edge and texture of such growths are to be particularly observed.

Colonies developed from the under surface of the gelatin, between the medium and the plate or dish, are usually in the case of aërobic species quite pale and thin though they may be spread over a considerable area. Facultative anaërobes do not show the same characteristic appearance.

Deep colonies in agar:

Deep colonies in a nutrient agar plate seldom show as marked or characteristic appearances as those in gelatin.

This medium never undergoes the change in consistence that occurs in gelatin, and is much firmer, thus preventing a characteristic development.

Surface colonies on agar:

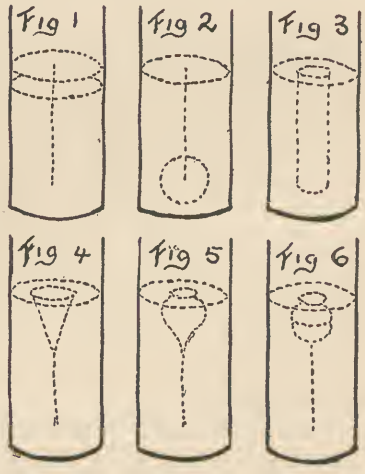
Upon the surface of agar, however, growth is much more apt to spread than it is upon gelatin on account of the condensation water which collects when the medium solidifies. A moderate amount of moisture allows of a natural spreading of the growth and occasionally of the development of very characteristic colonies.

Colonies in gelatin punctures:

Growths obtained in punctures in nutrient gelatin are so various and in many ways so characteristic that they should be accurately recorded.

As our media hold but a small quantity of free oxygen, aërobic growth occurs mostly on the surface, anaërobic growth entirely beneath the surface, and facultative anaërobes develop proportionately along the line of puncture, in the surface as well as in the deeper layers of the medium.

The growth of species which liquefy the gelatin is especially noteworthy, and



as types of this growth and liquefaction the following may be noted: viz., transverse liquefaction may be surface (Fig. 1), or deep (Fig. 2), or may extend throughout the length of the puncture producing a "stocking" or "glove finger" shaped liquefaction (Fig. 3). Liquefaction commencing on the surface may extend downwards in a "funnel shape" (Fig. 4), or when advancing more rapidly just beneath the surface may give rise to a "tulip" or "turnip" shaped fluidification (Fig. 5), and as liquefaction occurs there may occur a rapid evaporation of the water of the liquefied medium and the formation of an air buble as shown in Fig. 6.

5. *Action upon proteids, milk and serum.*

[See Chart].

6. *Action upon carbohydrates.*

Careful studies of the chemical action of bacteria upon the media in which they grow show that fermentation and gas production, in the presence of fermentable compounds, is perhaps the rule rather than the exception.

The compounds which lend themselves most readily to gas production and fermentation are the various forms of carbohydrates, and of these dextrose, lactose, and saccharose are the most useful for experimentation.

For the bacteriologist the simplest test of the occurrence of fermentation is the presence of a gas; and although gas may show itself in solid media as imprisoned bubbles or as clefts formed by the escaping gas, yet this gives no accurate information as to the amount or kind of gas formed by different species, under different conditions of growth and with different sugars. Here it is that the greatest value of the "fermentation tube" is shown, for the amount of gas formed can be readily recorded at stated times by a mark upon the closed branch or accurately measured with a millimeter scale; while the kind of gas may be determined by comparatively simple chemical tests.

For purposes of comparison it is important that the fermentation tubes used for all these determinations should be uniform in size, the amount of gas being, under these conditions, expressed in mm.[1]

Experience thus far shows that bacteria which are capable of producing fermentation, usually act upon two of the three sugars mentioned, and rarely upon but one or upon all three; and there seems to be no method except by the direct experiment to determine which two of the sugars will be acted upon by them. For this reason it is important that all species should be tested for their action upon each of these three carbohydrates.[2]

A difficulty met with in the use of meat or meat extracts as the nutrient basis of fermentation broth is the presence of muscle sugar, a compound

[1] In cases where the closed branches of fermentation tubes are uniform in calibre but vary in length, the amount of gas formed should be expressed in terms of the tube length, in percentages.

[2] For preparation of fermentation broths, see p. 77.

which chemically and in its fermentative reactions to the bacteria, seems to be identical with dextrose. When experimenting with dextrose the presence of muscle sugar is of no account, but in experiments with lactose or saccharose it is most important that this compound should be eliminated. To this end all broths should be tested when first made by inoculating a few fermentation tubes containing samples of it with well-known active gas-forming species and when gas is absent muscle sugar can not be present in the proportion of more than .03 per cent, which, according to Dr. Theobald Smith, is an amount which need not be taken into consideration.

The dextrose-free broth of Smith has already been described (p.78). To this the sugar should be added.

To inoculate any species, whether aërobic or anaërobic, in broth contained in a fermentation tube, it is necessary only to float a little of the culture in the fluid in the bulb. It is not necessary, nor is it desirable to carry the needle down to the connecting tube or into the closed branch, lest a certain amount of oxygen should be carried into the closed branch and thus vitiate part of the results it is desirable to obtain. In case of gas formation, at regular intervals, preferably at the end of every twenty-four hours for several days, the level of the fluid in the closed branch is marked upon the tube or measured with a millimeter scale, and the results recorded in percentages of the length of the closed branch; thus if 1 cm. of gas has formed in a closed branch 10 cm. in length 10 per cent. of gas is said to have formed. This is sufficiently accurate as only comparative values are considered.

The gases which are most commonly and abundantly formed in the fermentation of sugars are carbonic acid and hydrogen, and valuable information is afforded by determining their relative proportion in the column of gas.

To test the amount of CO_2 present a two per cent. solution of sodium hydroxid is employed. The bulb is completely filled with this solution, the thumb placed over the mouth of the bulb, and the fluid is run back and forth through the length of the tube six or eight times and the gas remaining again returned to the upper end of the closed branch. The thumb is now removed and the amount of gas remaining is measured in millimeters,—the difference between this and the former measurement shows in m. m. the amount of CO_2 absorbed by the alkali. The remaining gas may now be transferred to the bulb and exploded in a flame.

A practical method of recording the results of the gas tests will be best understood by referring to the chart at the end of this report. It is to be remembered that only the relative amount of gases formed is requisite for species determination, and that absolute determinations have not been sought for.[1]

[1] For methods of making analyses of gases formed during fermentation, reference must be made to text-books on gas analysis, *e. g.*, Hempel's Gas Analysis translated by Dennis, MacMillan & Co., 1892.

7. *Action upon nitrates.*

Among the principal changes produced in organic compounds by bacteria is their action upon nitrogenous substances. Of some diagnostic value is the determination of the capacity of certain bacteria to reduce nitrates to nitrites and ammonia. For this test a special medium, nitrate broth,[1] is required. Nitrites and ammonia are commonly found in the air and are readily taken up in solution by watery fluids, so that in testing for the presence of these substances it is requisite that a control tube containing nitrate medium which has not been inoculated, but which has been kept under exactly the same conditions as the inoculated tube, should also be tested for the presence of these compounds.

The inoculated tubes together with a sufficient number of uninoculated control tubes should be incubated at 20° C. for seven days and then submitted to the following tests for nitrites:

Prepare two test solutions:

I. Naphthylamin . . . . . 0.1 g.[2]
Distilled water . . . . . 20.0 c. c.

Boil until the naphthylamin is dissolved, then cool, filter, and add the clear filtrate to hydric acetate dilute (1 to 16 of water) 150 c. c.

II. Sulphanilic acid 0.5
Hydric acetate dilute . . . . . 150.0 c. c.

These clear solutions are best kept separate in glass bottles with closely fitting glass stoppers, and mixed in equal parts just before use.

About 3 c. c. of the culture or of the medium in the control tubes are poured into a perfectly clean test-tube, and to this is added gradually about 2 c. c. of the test solutions previously mixed in equal parts. The development of a red color indicates the presence of nitrites, the amount of nitrites being in direct proportion to the intensity of the color, therefore a pinkish or reddish color of about the same intensity developed in both the culture and uninoculated medium would indicate that a small amount of nitrites was present, either having been present in some of the ingredients used in making the medium or absorbed from the air; while a deeper shade of color in the culture would show the development of nitrites from the reduction of the nitrates in the medium. The formation of the color showing the presence of nitrites is sometimes delayed, but may be hastened by heat.

When this test shows the presence of nitrites, one half of the remaining culture or control fluid is to be tested with Nessler's solution[3] for the presence of ammonia, for the purpose of determining the extent to which reduction has occurred. The presence of ammonia is shown by the immediate development of a yellow color or precipitate on the addition of a few drops of the test solution.

[1] Pepton, gm. 1, potassium nitrate 0·2, water c. c. 1000.

[2] See Centralblatt für Bakt & Parasit. Bd. XVI, 1894, p. 945.

[3] For the preparation of Nessler's solution reference must be made to some text-book on chemistry.

When these tests are positive our inquiry as to whether or not the nitrates have been reduced is answered, but when negative, one of two conditions may be present, either the nitrates may have remained unchanged or may have been reduced to free nitrogen.

It is therefore necessary to determine whether nitrates are present or not, and for this purpose the phenolsulphonic acid and sodium hydroxid test is recommended,[1] showing the presence of nitrates by the production of a yellow color.

8. *Production of indol.*

Among the products formed by bacteria growing in media containing proteids is indol,[2] a substance which may be easily detected by tests, and which has largely been employed for species differentiation.

The culture fluid usually recommended for this test is the pepton and salt broth (Dunham's solution). It has, however, been shown by Theobald Smith (Journal of Experimental Medicine, Vol. ii, p. 543), and by Peckham (Ibid, vol. ii, p. 554) that the best results in determining the capacity of bacteria for the production of indol are not obtained by the use of Dunham's solution. It is important that carbohydrates should not be present in the culture fluid, as these inhibit the production of indol. Theobald Smith recommends the dextrose-free bouillon whose composition has already been given (p. 78). This gives good results, as does also the medium recommended by Peckham, for the composition of which the reader is referred to the article already cited. The reagents used in making the test are 0.01 per cent. solution of sodium (or potassium) nitrite, and chemically pure sulphuric acid. The date at which the largest amount of indol is present in the culture varies in different cases. The proportion in which the reagents are to be added in order to obtain the most intense color varies also in different cases, so that exact rules cannot be laid down as to either of these points. For further details the reader may consult the papers of Smith and of Peckham.

9. *Production of acid and alkali.*

The production of acid in culture media is of very wide occurrence among the bacteria and may be found under all circumstances, or be restricted to growth occurring only under certain conditions.

As pointed out by Theobald Smith, much information on this point may be obtained from an examination of growths in sugar broths[3] in fermentation tubes, it appearing that acid formation is largely wanting in those which grow only in the bulb, *i. e.*, obligatory aërobes, while among

[1] Vide Fuller. Differentiation of the Bacillus of Typhoid Fever. Boston Medical and Surgical Journal, September 1, 1892.

[2] Indol is not infrequently present in pepton and errors from such a source must be guarded against.

[3] In preparing these sugar broths it is important that dextrose-free bouillon be made as already described (page 78) and the desired sugar (usually glucose or lactose or saccharose) be added to this —without this precaution serious errors may be made in interpreting the results.

those species which grow in both the bulb and the closed branch, *i. e.* facultative anaërobes, the fluid in the closed branch is usually, perhaps always acid while that in the bulb, *i. e.*, exposed to the air, may prove to be either acid or alkaline. Anaërobes are usually active acid producers, especially in the presence of carbohydrates.

For the differentiation of species, therefore, the production of acid quantitatively with reference to any given carbohydrate, and qualitatively with reference to different carbohydrates, becomes of considerable importance. Qualitative tests may be made from day to day by withdrawing a drop of the fluid from both the closed branch and bulb of the fermentation tubes and testing their reaction to litmus paper, but this method is inexact, and to that extent unsatisfactory. A more satisfactory qualitative study may be made by cultivating the species under consideration in standard solid media to which have been added one per cent. of glucose, saccharose, or lactose, and a sufficient quantity of litmus solution to produce a blue tinge. If acid is produced as a result of the action of the bacteria upon the sugar the colonies assume a pale pink color and there may be also a reddening of the surrounding medium. Tests should be made with the three sugars mentioned.

For testing quantitatively the acid or alkali formed a measured quantity (say 5 c. c.) of the culture fluid from both the closed branch and bulb should be titrated with a 1-20 normal sodium hydroxid solution or a 1-20 normal hydric chlorid solution as may be required. A sample of the uninoculated broth must also be titrated to afford a basis for the compu tation of the changes induced.

10. *Pigment formation.*

The development of color in a colony or in the medium immediately surrounding the growth is one of the most obvious characters of many species of bacteria and is important as a means of species differentiation. Pigment may develop differently according to the composition of the medium, its acidity or alkalinity, as the growth is or is not exposed to the action of oxygen, the temperature at which growth occurs, and the period of its development.

To make this character an available one, therefore, in work upon species it is necessary:

1. That the composition and reaction of all media be accurately recorded in or upon which color develops.

2. That it be noted whether pigment is formed in the surface or deeper parts of the growth or medium, or in both.

3. That the temperature be given to which the culture forming pigment has been exposed and the period at which the pigment forms, and,

4. That such a description of the color produced be afforded as to render it recognizable to workers in other laboratories who may not have seen the culture in question.

To fulfill these requirements, it is suggested that the media here recommended as standard be employed, and that the composition and reaction of other media which may be used be accurately recorded, all reactions being referred to the neutral point of phenolphthalein; that both surface growths and deep punctures be employed; that growths at 18°–20° C. and at 36°–38° C. be studied; and that the color produced be recorded according to some color scheme which will serve all workers as a basis for identification. Such a scheme of colors has been devised by Dr. Shuttleworth, and is here appended.[1] For the proper use of these plates reference must be had to Shuttleworth's original article "On the Nomenclature of Colors for Bacteriologists," published in the Journal of American Public Health Association, p. 403.

Characters depending upon the disposition of light, etc.—(Shuttleworth):

Transparent.

Vitreous; transparent and colorless.

Oleaginous; transparent and yellow; olive to linseed oil colored.

Resinous; transparent and brown; varnish or rosin colored.

Translucent.

Paraffinous; translucent and white; porcelaneous.

Opalescent translucent, grayish-white by reflected light, smoky brown by transmitted light.

Nacreous; translucent; grayish white with pearly lustre.

Sebaceous; translucent; yellowish or grayish white; tallowy.

Butyrous; translucent and yellow.

Ceraceous; translucent and wax colored.

Opaque.

Cretaceous; opaque and white; chalky.

Dull; without lustre.

Glossy; shining.

Fluorescent.

Iridescent.

11. *Development of odor.*

As no method of analyzing or recording odors has been devised or seems probable, accuracy of record of odors produced is impossible. The odors developed by many species of bacteria, however, are peculiarly characteristic, and although some defy description, quite a number may be more or less accurately likened to odors evolved by well-known organized material under various conditions, or with specific odors of sundry chemical compounds. The fact that certain species develop odor in all media or only in media of certain composition, or fail to develop any odor at all, is important, and should be accurately ascertained and recorded.

[1] On account of the cost of reproduction this color scheme had to be omitted from the printed report.

c. Pathogenesis.

It may properly be questioned whether the very obscure and variable reactions on the part of the living organism towards bacteria and their products ought strictly to be included in any scheme of exact tests for species differentiation. Again, it is a matter of common knowledge that of all features, the pathogenic properties of at least many forms of bacteria are those which are capable of the greatest amount of variation, and this fact also is against the employment of the test of inoculation for determining the affinities of any bacteria under consideration. But, granting all this, it has further to be acknowledged that up to the present time a main object of bacteriologic study has been in the direction of determining the relationships of bacteria and their products towards man and the higher animals, or of studying the relationship of bacteria towards organized products.

Hence, a study of the pathogenic properties of bacteria assumes a peculiar importance, and the Committee feel it necessary to recommend the adoption of the test of pathogenesis as a routine procedure in the determination of the characters of all so-called species of bacteria.

The necessary procedure is much simplified by the fact that while many forms grow at the temperature of the blood of warm-blooded animals, a large number of others will not grow at so high a temperature. The products of these latter species, however, may possess toxic properties; and, as throwing light upon this point, inoculations of older cultures should be made in some animal. Therefore,

1. Where a given form grows only at or below 18° to 20° C. inoculation should be made, into the dorsal lymph-sac of a frog, of about 1 per cent. of the body weight of the frog of a liquid culture seven days old.

2. Where a given species grows at 35° C. or upwards an inoculation should be made into the peritoneal cavity of the most susceptible (in general) of warm-blooded animals, *i. e.*, the mouse, either the white or the ordinary house mouse; the amount to be inoculated should be about 1 per cent. of the body weight of the mouse, of a 48-hour standard broth culture, or a broth or watery suspension[1] of one platinum loop (see p. 86) from solid cultures. When such intraperitoneal injection fails it is unlikely that other methods of inoculation will be successful in causing the death of the mouse.

If the inoculations of the frog and the mouse both prove negative the Committee think it unnecessary to insist upon any further tests upon pathogenesis, as being requisite for work in species differentiation, as strongly pronounced pathogenic properties if present would most likely have shown themselves from the previous inoculations.

If, on the other hand, the mouse succumbs to the inoculation, there should be appended either to the original description or forming the subject of a separate research, the results of inoculation of the species under

[1] One loopful in 5 c. c. of liquid.

consideration into other animals, more especially into the rabbit and the guinea pig. The methods of inoculation are given in text-books on bacteriology. The ordinary hypodermic syringe will answer for inoculations. It should be kept in a 2 per cent. solution of carbolic acid and thoroughly washed out five or six times with sterilized water, broth, or other indifferent fluid, just before use.

It is not advisable to employ for subsequent experimentation animals which have survived inoculation, as but little is known of the influence which a previous inoculation may have upon the susceptibility of the animal to the same or to other species of bacteria.

After inoculation the animal should for some days be kept under observation, all signs of disorder should be noted, and in the case of larger animals the rectal temperature recorded at least twice in each twenty-four hours. When the animal has appeared to have survived the inoculation it should be kept under observation for not less than a month.

If the animal dies, a most careful autopsy should be made as soon as possible after death; the site of subcutaneous inoculation should be opened up and cultures, always in duplicate, should be made from here as well as from the peritoneal and pleural cavities, the spleen, liver, kidney, lung, and heart's blood. The autopsy should be made under the strictest antiseptic precautions and the surface of each viscus should be seared with a red-hot spatula or other metal before obtaining the material from which the cultures are to be made. Tissue from the site of the inoculation and from the viscera mentioned should be subjected to microscopic examination to determine histological changes or the presence of bacteria, and in every instance a number of so-called "tissue smears" on the cover-glass should be examined. The procedure for determining the kind and number of bacteria present is by the plate method, and although no single medium can be said to be sufficient for these determinations, the one which generally best answers the purpose is nutrient glycerin agar. It is important to distinguish between actual infection and intoxication resulting from the injection of the products of bacterial growth along with the bacteria. This distinction is not always easily made but where large doses are required to produce symptoms or to cause death, there should be a strong suspicion of pure intoxication and especial attention should be given both to the distribution of the bacteria in the animal body and to evidences of multiplication of the bacteria in the system.

While fully alive to the importance which attaches to the toxicity of the products of species, which are not directly infectious and pathogenic the Committee feel that they should not demand as necessary tests, the possible toxic products derived from the bacteria or from the media in which they are grown, apart from the presence of the living bacteria.

For a fuller discussion of Pathogenesis see Welch, "What shall be the mode of procedure in determining the pathogenesis of bacteria found in water?" Proceedings Bacteriological Convention *Loc. Cit.* p. 502.

OPTIONAL TESTS OF GENERAL USEFULNESS.

It is difficult to know how much or how little to say in reference to the tests which have been brought together under this heading. It must be fully understood that as the morphological characters of these minute organisms offer such an insufficiency of characters on which to base a differentiation of species that every biological character, whether positive or negative, is of aid in this regard, and it is far from the wish of the Committee to limit the amount of careful study which may be applied to bacterial forms; but on the other hand they believe that they can best perform the work which they have been asked to do, by making clear to those engaged in the study and differentiation of species of bacteria the absolute need of recording the results of at least a certain minimum number of features in connection with each form.

Among the tests classed as optional (see p. 61) there are some to which fuller attention may be drawn and which perhaps in the near future, when more fully understood, may be regarded as tests of primary importance.

I. MORPHOLOGICAL.

3. *Permanence of morphological characters.*

Experimentally it is not a difficult matter to vary the properties of certain species of bacteria to such an extent, that were we given the modified form without information as to the previous treatment it had been subjected to, it would be an easy matter to believe that we were dealing with a species different from the parent organism. How then are we to be assured that many of the closely allied forms which are encountered in the bacterial study of water, air, soil, etc., and in the bodies of diseased patients, are truly distinct species?

It must be confessed that at the present time it is impossible to give anything like a complete answer to this question; nevertheless, in a certain number of cases it would seem that continued growth, under what may perhaps be termed more normal conditions, does lead forms which appeared to be distinct, to approximate more closely to each other and to a common type.

This is especially noticeable in the so-called experimental varieties; forms which by modification of temperature or environment have departed widely from the original, tend when grown upon ordinary media and under ordinary laboratory conditions, to revert, sooner or later, to the characteristics of the original laboratory form.

This being so, it would seem that important information may be gained as to the relationship of any form by successive growths upon the ordinary standard media for not less than a year, control and parallel growths of the form or forms to which it appears to be allied being carried on at the same time and on the same media; and it may further be recommended that all who describe new species be urged to publish a second description after a period of 12 months has elapsed, in the same journal

in which their first communication appeared, this second description to state accurately how far the forms have become modified by continued growth on the standard media. It is important that a uniform method of treating the cultures during this period be observed and to this end the following is recommended :

Obtain approximately the maximum growth on standard nutrient agar (see p. 80) at 36°–38° C. (or at 18°–20° C.) and then place in a refrigerator at about 12°–15° C. Resowings should be made every 4 or 6 weeks.[1]

4. *Photographic reproductions of isolated bacteria.*

Inasmuch as it is not always possible to procure the publication of photographs, the Committee have not classed this as one of the requisites in describing species, yet, when possible, photographs of the isolated bacteria, grown under definite conditions, should be published, care being taken that the exact magnification is given. In any event such photographs should always be taken from which "blue prints" could be made.

What is said here with regard to reproducing photographically the isolated bacteria is equally true with reference to photographing characteristic growths on plates (surface and deep colonies) and in tubes. It goes without saying that photographs are far more trustworthy records than drawings.

II. PHYSIOLOGICAL.

a. Cultural characteristics.

3. *Synthesised media.*

The variation in chemical composition of many of the ingredients employed in preparing nutrient media, more especially in the organic compounds, will necessarily lead to a diversity in description which may occasion confusion.

Synthesised media, *i. e.*, media prepared from compounds of exact chemical composition, have been largely experimented with, but thus far with unsatisfactory results.

No such medium has been devised on which even the majority of bacteria will grow satisfactorily and on most of the synthesised media recommended there is a loss of characteristic features such as pigment formation, etc.[2]

A practically useful synthesised medium is a great desideratum in the interest of scientific accuracy in differentiating species.

b. Biochemical features.

1. *Temperature of growth.*

Among the necessary tests are included the activity of growth at only two temperature ranges and the determination of the thermal death point

[1] See Adami—"How is variability in bacteria to be regarded?" Proceedings Bacteriological Convention, *Loc. Cit.* p. 415.

[2] See MacKenzie—"What new methods can be suggested for the separation of bacteria into groups and for the identification of species?" Proc. Convent. *Loc. Cit.*, p. 419.

(see p. 85) but for the accurate determination of temperature relationship a study should also be made of the minimum and maximum temperatures at which growth can occur and the temperature at which growth is most prolific, *i. e.*, the optimum growth temperature. It may be stated that the apparatus absolutely required for these determinations is comparatively moderate, as much information may be gained by the use of a few water-baths, fitted with thermostats and a moderately good refrigerator.

4. *Chemistry of bacterial pigments.*

Very little has yet been accomplished in this branch of bacteriology. It would seem, however, from what has been learned that fuller studies of the reaction of the pigments produced and of their solubility, together with spectroscopic observations upon them, might afford valuable information as to the relation existing between various forms.

c. Pathogenesis.

The tests of pathogenesis which are included among the necessary tests the Committee consider far from complete, and would here briefly urge that these tests be conducted upon more than one animal of a given species, and upon animals of different species; that whenever inoculation leads to the production of definite lesions, a careful study be made of the local and general changes produced in the system; and that where a form is found to be pathogenic, further researches be made as to the immunity producing properties of the organism and its products.

Lastly, the researches of the past few years have shown an importance in determining and isolating the toxic substances, not only free from cultures of pathogenic, but also from those of non-pathogenic, species.

A study of toxic substances and their properties would seem to promise information of so much value not only with regard to the nature of disease, but also as to the evolution of pathogenic bacteria, that it is strongly recommended that account be taken and studies be made of the toxic substances of bacterial origin.

The studies in recent years of Pfeiffer, Gruber, Widal, and others upon the specific properties of the blood serum and other humors of animals and human beings during the period of infection or that of acquired immunity, have demonstrated that these properties may be of great value in the diagnosis of disease and in the differentiation of bacterial species. The Committee would urge that use be made of these properties of immune sera for the differentiation of bacterial species, wherever they are available, but in the present state of the subjects and within the limits of this report it is not deemed necessary to give directions as to the requisite procedures or to discuss their precise value. Information upon these points is readily accessible in the special journals and works relating to bacteriology.[1]

[1] Consult especially Pfeiffer, Zeitschrift für Hygiene u. Infections Krankheiten, Bd. XVII, XVIII, XIX, XX, Pfeiffer and Kolle, Ibid, Bd. XXI. Gruber, Archiv für Hygiene, Bd. XX. Münchener Med Wochenschrift, 1896, p. 206, and 1897, Apr. 27 and May 4. Widal and Sicard, Annales de l' Institut Pasteur, 1897, and Flügge Die Mikroognaismen, 3te Auflage, Leipzig, 1896.

STANDARD CHART FOR BACTERIAL DIAGNOSIS.

Compiled by T. M. CHEESMAN, M. D.,

Instructor in Bacteriology, Columbia University, and Adopted by a Committee of American Bacteriologists, October, 1897.

No..... Date.....

Source..... Habitat.....

Morphological examination of agar culture grown ..... days at 18°–20°C.; ditto grown ..... days at 36°–38°C.
" " gelatin culture grown ..... days at 18°–20°C.
" " broth " ..... " " ; ditto grown ..... days at 36°–38°C.
Micrococcus, single, irregularly grouped; diplococcus; streptococcus; tetracoccus; sarcina.
Bacillus, single; in chains; in filaments.
Spirillum, comma; spiral.

Size, in terms of blood cell / ; breadth.....μ. Average length.....μ. Extreme lengths from .....μ to .....μ.

Stain, with standard watery dyes easily or with difficulty, uniformly or irregularly.
by Gram's method decolorizes or does not decolorize.

Capsule, none observed or may be easily observed or may be demonstrated and stained by Ziel's, Gram's, or Welch's method.
conditions under which it is most likely to be present are ..... Broad or narrow.
demonstrable or not demonstrable in serum, milk, or surface agar cultures.

Spores, none observed or do form within .... hours at .... °C. on standard .....; are located centrally, or on the end; do or do not give rise to enlargement of bacterial cell, causing clostridium or drum-stick forms; germinate within .... hours at .... °C. Stain by Moeller's or Abbott's method; are killed at 100°C. within ..... minutes; withstand 100°C. for ..... minutes. Germination inhibited by this latter exposure for ..... days. Germination observed or not observed.

Vacuoles, none observed or are observed on standard .....

Crystals, none observed or are observed on standard .....; dissolved by chloroform or ether.

Motility, not observed (or brownian movement) or is observed; sluggish or active, rotary or direct, most pronounced in cultures obtained from ....., grown for .... hours at ....°C. Flagella stain by Loeffler's, Van Emengemi's, or Bunge's method; originate from one or both ends, or irregularly from the periphery; single or in tufts (monotrichal, lophotrichal, amphitrichal, or peritrichal).

Pleomorphism, not observed or is observed on standard ..... at .....°C., or on media reacting ..... per cent. acid or ..... per cent. alkaline when grown at .....°C. for ..... days.

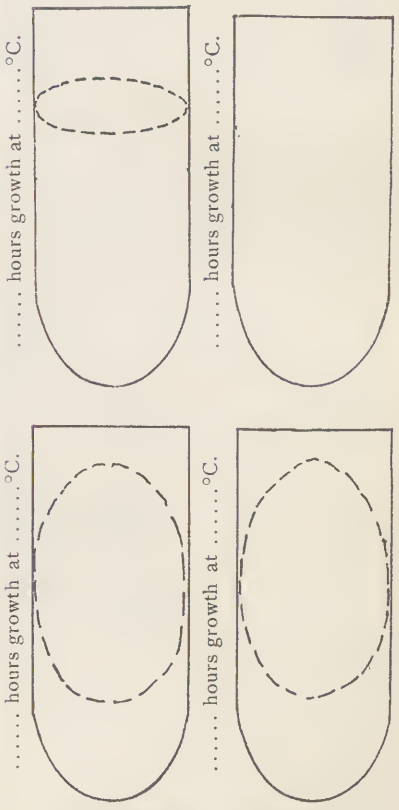
Growth at 18°–20°C. is more abundant or less abundant than that at 36°–38°C.
Thermal death point .....°C; time of exposure ..... minutes. Permanence of morphological characters .....
Optimum temperature .....°C. .....
Optimum reaction of media from ..... per cent. to ..... per cent. ..... Growth limits, maximum .....°C.; minimum .....°C.

		Deep Colonies.	Surface Colonies.
Gelatin plate. Size. Shape. Margin. Texture. Color. Growth under mica plate present or absent.	Reaction of medium.		
Agar plate. Size. Shape. Margin. Texture. Color. Growth under mica plate.	Reaction of medium		
Gelatin tube. Puncture. Depth. Form. Consistence. Surface growth. Deep growth. Streak. Size and shape. Margin. Surface relief. Light transmission. Light reflection. Color. Lustre. Change in medium. Color. Consistence. Odor.	Reaction of medium.		
Agar tube. Streak. Extent and shape. Margin. Surface relief. Light transmission. Light reflection. Color. Lustre. Change in medium. Color. Transparency.	Reaction of medium.		

Sketch of Germ and Colony.

Gelatin and Agar.

Tube Cultures.



Nutrient broth in test tube.

Remains clear.

BODY OF LIQUID.

Remains clear after ..... days at .....°C.
Growth appears near surface in ..... hours at .....°C.
" forms deposit in ..... hours at .....°C.
deposit is granular or flocculent and disseminated through the fluid.

SURFACE OF LIQUID.

Pellicle, does or does not form.
thickness in ..... hours at .....°C.
color in ..... hours at .....°C.
consistence in ..... hours at .....°C.
structure when fully formed after ..... days.
Deposit, forms in ..... hours at .....°C.
amount in ..... days at .....°C.
color in ..... days at .....°C.
character, whether compact or flocculent.
on agitating appears granular, flaky, or viscid.
Odor does or does not develop.
Reaction of culture at end of 1 day ....., ... days ....., ... days ....., ... days ....., ... days ....., 21 days .....

Becomes clouded.

Is opalescent or turbid in ..... hours at .....°C.
Clears on standing after ..... hours.
On shaking forms masses which may be fine, granular, or flocculent.

Becomes colored.

In ..... hours at .....°C.
Different color at .....°C.
Color uniform throughout.
" differs in surface and deeper layers.
" modified by shaking.
Colors..... in medium reacting ..... per cent acid.
" ..... " " ..... per cent. alkaline.

Broth and Milk.

Milk.

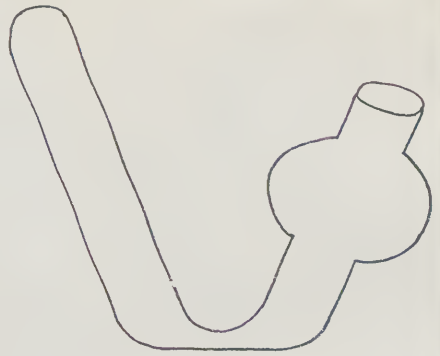
No visible change in ..... days at .....°C. Reaction in ..... days. Does or does not curdle on boiling.
Does not curdle in ..... days at 16°–18°C.
" " ..... " 36°–38°C.
Reaction .....
Does or does not curdle on boiling.
Color is or is not developed.
Gas is or is not developed.
Digestion, becomes gradually transparent without forming curds.
Transparency complete or incomplete in ..... days at .....°C.
Reaction after change has occurred.
Effect of boiling after change has occurred.
Odor is or is not developed.

Does curdle in ..... days at 16°–18°C.
" ..... " 36°–38°C.
Reaction when curd forms ..... per cent. .....
in 14 days ..... per cent. ....., in 21 days ..... per cent. .....
Color is or is not developed.
Curd, hard or soft, in one mass or in small fragments.
fragmented by gas bubbles or smooth.
changed or unchanged on boiling.
Whey, separated from curd or not.
amount, transparent or turbid.
Digestion. Curd remains unchanged or is dissolved.
Solution is rapid or slow.
complete or incomplete in ..... days at .....°C.
Reacts ..... per cent. ....., is clear or cloudy.
watery or viscid.
Odor is or is not developed.
Reaction at end of process, in ..... days at 16°–18°C.
" ..... " 36°–38°C.

Sugar broth in fermentation tubes.

	Amount of gas in per cent. formed in 1 day.	2 days.	3 days.	4 days.	.... days.	Reaction to litmus paper of fluid in bulb.	CO_2 per cent.	H per cent.
At 16°–18°C.								
G.	.....	.....	.....	.....	.....	.....	.....	.....
L.	.....	.....	.....	.....	.....	.....	.....	.....
S.	.....	.....	.....	.....	.....	.....	.....	.....
At 36°–38°C.								
G.	.....	.....	.....	.....	.....	.....	.....	.....
L.	.....	.....	.....	.....	.....	.....	.....	.....
S.	.....	.....	.....	.....	.....	.....	.....	.....

G=Glucose. L=Lactose. S=Saccharose.



In bulb, pellicle forms or does not form.
remains clear, is granular or flocculent.
becomes clouded, fluid is opalescent or turbid.
clears on standing in ..... hours.
color develops or does not develop.
on surface.
uniformly throughout.
in deeper layers only.
In connecting tube, growth forms deposit, is granular or flocculent.
color does or does not develop.

In closed branch, growth does or does not develop.
remains clear, growth is granular or flocculent.
becomes clouded, in upper or lower layers, or uniformly throughout.
clears on standing in ..... hours.
color does or does not develop.

Lactose litmus, agar or gelatin.

Reaction neutral to litmus.

Color changes to ..... in ..... hours at .....°C.
" " ..... " ..... " .....°C.
" " ..... " ..... days at .....°C.
Decolorized in ..... hours or days in upper or lower layers.
" in ..... days throughout at .....°C.

Gas does or does not develop.
Growth more or less abundant than on nutrient gelatin or agar.
Color does or does not develop.

Potato.

Reaction to phenolphthalein .... per cent. acid.

Growth occurs or does not occur.
apparent or not apparent.
restricted or spreading.
shiny or dull.
with or without lustre.

Gas does or does not develop.
Color does or does not develop in growth.
in medium.

Blood serum. Liquid. Character of growth.
Solidified. " " . Is or is not liquefied.

Nitrate broth. Nitrate is or is not reduced. Nitrites, ammonia, or free nitrogen present.

Dextrose-free bouillon. Indol is or is not produced.

Sugar media. Blood serum. Nitrate broth. Pepton and salt solution.

Pigment, is or is not developed ; in presence or absence of oxygen.
develops in ..... medium, in ..... days at .....°C., medium reacting ..... per cent. .....
Color No. ..... Changes to No. ..... by addition of acid or alkali.
Soluble in .....
Spectrum analysis shows .....

Production of acids or alkalis. Carbohydrates absent.
" present.

Relation to free oxygen. Obligatory aërobe.
Facultative anaërobe.
Obligatory anaërobe.

Relation of growth to acidity or alkalinity.
Per cent. alkali 4.
3.
1.5
0.
Per cent. acid 0.5

Pathogenesis

Synthesised media.

Pigment. Relation to oxygen.
Alkali or acid production. Pathogenesis.
Synthesised media.